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(54) Title: CONJUGATED SURAMIN OR DERIVATIVES THEREOF WITH PEG, POLYASPARTATE OR POLYGLUTAMATE FOR CANCER TREATMENT

(57) Abstract

The present invention provides an assay that identifies compounds which inhibit cleavage of HGF/SF by serum proteases such as uPA, and methods in which such compounds are provided to reaction solutions, to cultured cells *in vitro*, or to a mammal *in vivo*, to inhibit cleavage of HGF/SF and to inhibit chemical and biological effects resulting from the activation of c–Met receptor by HGF/SF. The invention also provides methods for modifying suramin and suramin–related polysulfonated compounds that inhibit HGF/SF cleavage, by attaching PEG or polyanions such as polyglutamate or polyaspartate to the compounds to reduce cellular uptake of the compounds, thereby reducing their cytotoxicity. Also provided are a pharmaceutical composition containing at least one polysulfonated HGF/SF cleavage–inhibiting compound other than suramin, and a pharmaceutical composition containing at least one HGF/SF cleavage–inhibiting form of suramin or a suramin–related polysulfonated compound that is modified by conjugation to a chemical moiety that reduces uptake of the compound into cells. The present invention further includes methods wherein such pharmaceutical compositions are administered to a mammal with a tumor that is stimulated to grow by HGF/SF, to inhibit the growth or metastasis of the tumor in the mammal.

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CONJUGATED SURAMIN OR DERIVATIVES THEREOF WITH PEG, POLYASPARTATE OR POLYGLUTAMATE FOR CANCER TREATMENT

BACKGROUND OF THE INVENTION

1. Field of the Invention

The present invention relates to suramin-like and polysulfonated compounds which inhibit cleavage and activation of hepatocyte growth factor/scatter factor (HGF/SF), and which inhibit tumor growth and metastasis. The present invention also relates to methods of using such suramin-like and polysulfonated compounds to inhibit HGS/SF cleavage/activation *in vitro* and *in vivo*, and to methods for inhibiting tumor growth and metastasis in a mammal by administering a composition containing such a suramin-like or polysulfonated compound to a mammal in need of such treatment.

2. Description of Related Art

Suramin is a polysulfonated naphthylurea first used for the treatment of onchocerciasis and trypanosomiasis in the 1920's (Hawking, 1978). In the late 1970's, suramin was shown to act as a potent inhibitor of reverse transcriptase from several animal retroviruses (De Clerq, 1979), and was the first molecule evaluated for antiviral therapy in AIDS patients (Mitsuya et al., 1984). However, trials of suramin as an anti-HIV agent were abandoned due to adverse side effects. Renewed interest in suramin came about when it was shown to possess some anti-tumor properties (Myers et al., 1992). Studies with tumor cell lines *in vitro* have demonstrated that suramin has significant anti-tumor activity against a variety of tumor types, including prostate, breast, ovarian, and lung cancer (Finley, 1994).

In clinical trials, administration of suramin to give serum concentration of 250-300 μg/ml has resulted in anti-cancer effects in patients with adrenocortical cancer, adenocarcinoma of the kidney, T-cell leukemia lymphoma, and metastatic and hormone-refractory prostate cancer (Finley, 1994). Despite showing promise in the treatment of some human cancers, the dosage of suramin required for anti-tumor effects remains associated with numerous undesirable side effects. Reported side effects of suramin include malaise and fatigue associated with weight loss and anorexia, proteinuria, coagulapathy, adrenal

insufficiency, neuropathy, and neurologic, renal, cutaneous, lymphopenia, anemia, ophthalmologic and alopecia toxicities, many of which are reversible upon termination of treatment (Finley et al., 1994; Eisenberger et al., 1995).

An additional side effect of suramin is a marked perturbation of the lysosomal system which is similar to that observed in patients with inherited mucopolysaccharidosis (Gritli et al., 1993; Christensen et al., 1988; Rees et al., 1986; Constantopoulos et al., 1982; Rees et al., 1982). Interestingly, this effect depends upon the presence of serum albumin and is not observed under serum-free conditions (Baghdiguian et al., 1991). This was later shown to be due to altered intracellular localization of suramin-albumin complexes compared with non-complexed suramin (Baghdiguian et al., 1996). Suramin's relatively long half-life *in vivo* of 40 to 50 days (Finley, 1994) or longer (Strum et al., 1995) appears to be due, at least in part, to its binding to and slow release from plasma proteins (Finley et al). Hence, both the pharmacological and pharmacokinetic properties of suramin *in vivo* are dramatically altered by association of suramin with proteins present in blood (Muller et al., 1976).

Toxicity of certain proteins and chemical therapeutic compounds can be reduced by attachment of polyethylene glycol (PEG), which increases the compound's solubility, reduces its immunogenicity, and increases the stability and circulating life of the compound (Enzon, Inc., 1997). Suramin already has a long half-life *in vivo* and its toxic effects do not appear to be related to immunogenicity or insolubility, and there is no suggestion in the published literature that conjugation of PEG to suramin world reduce its toxicity or improve its therapeutic or inhibitory efficacy.

Attachment of polyglutamate to a molecule reduces the molecule's ability to cross, or to undergo transport across, a cell membrane (Moran, 1998); however, the prior art does not teach or suggest modifying suramin by conjugating it to polyglutamate or to a similar polyanionic moiety in order to lower its ability to enter cells, and so reduce its cytotoxicity.

The biochemical, cellular, and physiological mechanisms though which suramin mediates its various biological effects remain unclear. Suramin is known to inhibit many different types of enzymes in addition to inhibiting lysosomal enzymes, including

hexokinase, Na^+-K^+ ATPase, and DNA and RNA polymerases (Cristiani et al., 1995). Suramin also inhibits the binding of a variety of growth factors to cell surface receptors, and it is generally assumed that this inhibition of growth factor activity represents a major means through which suramin displays anti-tumor behavior. Growth factors inhibited or inactivated by suramin include platelet-derived growth factor, transforming growth factors, fibroblast growth factor, epidermal growth factor, insulin-like growth factor, interleukin-2, and hepatocyte growth factor/scatter factor (HGF/SF) (Finley, 1994; Cristiani et al., 1995). Specific mechanisms which have been proposed to account for suramin's anti-cancer activities include interaction of suramin with a growth factor nucleotide binding site (Doukas et al., 1995), suramin-induced shedding of HGF/SF receptors from cells (Galvani et al., 1995), inhibition of cellular enzymes required for folate metabolism (McGuire et al., 1996) or for critical nuclear functions (Fujiuchi et al., 1997), and inhibition of angiogenesis (Gagliardi et al., 1992; Cristiani et al., 1995).

HGF/SF is the ligand for the tyrosine kinase receptor encoded by the c-Met proto-oncogene (Bottaro et al., 1991; Naldini et al, 1991). It is produced and secreted by cells of mesodermal origin and acts on epithelial and endothelial cells, and its biological activities on different cell types include mitogenesis, morphogenesis, motogenesis or "cell scattering", cell invasiveness, and angiogenesis. Observation of these activities led to the proposal that HGF/SF is involved in tumor establishment, progression, and metastasis (Cristiani et al., 1995). Moreover, numerous studies implicate aberrations in the HGF/SF-Met signaling system in the etiology and progression of a variety of malignant tumors (reviewed in Jeffers et al., 1996). Co-expression of Met and HGF/SF in the same cell results in both tumorigenic and metastatic properties *in vivo* (Rong et al., 1992; Rong et al., 1994; Jeffers et al., 1996b; Jeffers et al. 1996c), and there are several reports showing that over-expression of Met and/or HGF/SF is associated with the development of tumors, particularly those of an aggressive phenotype (Ferracini et al, 1995).

In recognition of the important role that HGF/SF appears to play in the development of various types of tumors, there is a need to develop inhibitors of the HGF/SF-Met signaling

system, both to further elucidate the role of HGF/SF in tumorigenesis, and to block HGF/SF-stimulated tumor growth and metastasis where it occurs in cancer patients.

Suramin is an inhibitor of activation of the c-Met receptor by HGF/SF, as noted above. Both HGF-induced autophosphorylation of the c-Met receptor and HGF induced cell scattering are blocked when cells are exposed to suramin (Ferracini et al., 1995). Suramin-like polysulfonated derivatives of distamycin A having 2, 4, or 6 sulfonic groups also inhibit HGF/SF-induced c-Met receptor activation, and their ability to do so appears to depend strongly on the specific locations of the sulfonic groups in the compound, and on the structure of the molecular "backbone" which maintains the spatial geometry of the charged groups (Cristiani et al., 1995). It has been proposed that suramin binds to the ligand, i.e. HGF/SF, rather than to the receptor (Cristiani et al., 1995); however, specific biochemical effects resulting from the action of suramin and suramin-like compounds that account for the ability of these compounds to inhibit HGF/SF had not been identified, until the present invention.

HGF/SF is synthesized as a 90 kDa single chain precursor polypeptide (pro-HGF/SF) which is devoid of biological activity. The critical step in HGF/SF activation is proteolytic cleavage generating an αβ heterodimer in which an α chain of 60 kDa and a β chain of 32-36 kDa are bound to one another by a disulfide bridge (Vigna et al., 1994). Cleavage of pro-HGF/SF occurs in the extracellular environment through the action of extracellular proteases present in serum. Serum proteases which have been reported to cleave and activate HGF/SF include urokinase (Naldini et al., 1992), HGF-activator (Miyazawa et al., 1993), and HGF-converting enzyme (Mizuno et al., 1994). Thus, cleavage/activation of pro-HGF/SF represents the initial stage of HGF/SF-Met activation, and provides a possible point for interference by potential inhibitors.

BRIEF SUMMARY OF THE INVENTION

The present invention is based on the discovery that suramin and related polysulfonated compounds inhibit cleavage of pro- HGF/SF by serum proteases such as uPA. The invention provides an efficient assay for identifying inhibitors of HGF/SF activation by

testing compounds for their ability to inhibit serum-mediated cleavage of HGF/SF. The invention also identifies suramin-like polysulfonated compounds that inhibit HFG/SF according to the present invention, and provides methods whereby such compounds are used to inhibit HGF/SF activation by uPA and other serum proteases *in vitro* and *in vivo*.

The different levels of inhibitory activity shown by the compounds of the present invention support the concept that their ability to inhibit HGF/SF activation is structure-specific and depends strongly on the specific spatial arrangement of the charged sulfonic groups in each inhibitory compound.

The present invention further includes attaching to suramin and other polysulfonated HGF/SF cleavage-inhibiting compounds chemical groups that reduce cellular uptake of the compounds, and hence reduce their cytotoxicity. Among the chemical groups which can be attached to suramin and other polysulfonated HGF/SF cleavage-inhibiting compounds to reduce their cytotoxicity are PEG and polyanionic moieties such as polyglutamate or polyaspartate. Methods are provided for modifying suramin and related polysulfonated compounds by attaching such cell uptake-reducing groups to produce compounds of the present invention which are also potent inhibitors of HGF/SF cleavage. The modified compounds are not taken up into cells as efficiently as the unmodified compounds, and so are less cytotoxic. Furthermore, the modified compounds of the present invention remain in the extracellular environment where cleavage of pro-HGF/SF occurs, and so have a longer inhibitory half-life *in vivo*.

Since the modified suramin and suramin-related compounds of the present invention inhibit serum-mediated cleavage of HGF/SF, as shown by the disclosed assay of the present invention, they retain activity as inhibitors of the HGF/SF: c-Met signaling pathway, and so possess anti-tumor activity. Accordingly, the present invention further provides suramin and suramin-like polysulfonated compounds that are chemically modified so as to have reduced cell-penetrating properties and reduced cytotoxicity, which compounds inhibit tumor vascularization, growth, and metastasis. It further provides compositions containing such compounds, and methods in which compounds or compositions of the present invention are

provided to a mammal with a tumor that is stimulated to grow by HGF/SF, in order to inhibit the growth of the tumor.

Other objects and advantages of the invention will appear from the following description.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 depicts the inhibition of serum-mediated cleavage of pro-HGF/SF by suramin and heparin in the presence of the HGF/SF-producing cells. Proteins of S114 cells were labeled overnight with ^{35}S in the presence of 5% serum alone (lane 1), or serum plus 500 μg/ml heparin (lane 2), or serum plus 100 μg/ml (~70 μM) suramin (lane 3). ^{35}S-labeled HGF/SF polypeptides in the cell culture supernatants were collected, immunoprecipitated, separated by SDS-PAGE, and detected by autoradiography.

Figure 2 depicts the inhibition of serum-mediated cleavage of pro-HGF/SF by suramin and heparin in a cell-free solution. Cell culture supernatant containing ^{35}S-labeled proteins secreted by S114 cells was incubated overnight in the absence of serum (lane 1), or in the presence of 5% serum (lanes 2-4) either alone (lane 2) or plus 500 μg/ml heparin (lane 3) or 100 μg/ml (~70 μM) suramin (lane 4). ^{35}S-labeled HGF/SF polypeptides were then immunoprecipitated, separated by SDS-PAGE, and detected by autoradiography.

Figure 3 depicts the inhibition of urokinase (uPA)-mediated cleavage of pro-HGF/SF by suramin and heparin in a cell-free solution. Cell culture supernatant containing ^{35}S-labeled proteins secreted by S114 cells was incubated overnight in the absence of uPA (lane 1), or in the presence of 2 μg (~160 IU) of high molecular weight uPA (lanes 2 & 3) either alone (lane 2) or in the presence of 100 μg/ml (~70 μM) suramin (lane 3). ^{35}S-labeled HGF/SF polypeptides were then immunoprecipitated, separated by SDS-PAGE, and detected by autoradiography.

Figure 4 depicts the synthesis and structure of a PEG-suramin ester. Suramin free acid, PEG-400, and 4-dimethylaminopyridine (DMAP) are dissolved in dimethylformamide (DMF), a solution of dicyclohexylcarbodiimid (DCC) in chloroform is added at room temperature, the reaction mixture is stirred at room temperature. Subsequent filtration, evaporation, extraction, and drying steps yield PEG-suramin esters where n is 1-6. For the PEG-suramin ester product shown in the figure, n is 4.

Figure 5 depicts the inhibition of serum-mediated pro-HGF cleavage by PEG-suramin ester in a cell-free solution. Cell culture supernatant containing ^{35}S-labeled proteins secreted by S114 cells was incubated overnight in the absence of serum (lane 1), or in the presence of 5% serum (lanes 2-4) either alone (lane 2) or plus 200 µg/ml PEG-suramin (lane 3). ^{35}S-labeled HGF/SF polypeptides were then immunoprecipitated, separated by SDS-PAGE, and detected by autoradiography.

Figure 6 depicts the chemical structures of suramin and other polysulfonated compounds of the present invention.

Figures 7A and 7B depict the abilities of suramin and other polysulfonated compounds of the present invention to inhibit serum-mediated cleavage of pro-HGF/SF. 1 µg of human HGF/SF produced by S114 cells under serum free conditions was incubated overnight in the absence of serum (lanes 1), or in the presence of 5% FBS (lanes 2-8) alone (lanes 2) or plus each of the compounds shown in Figure 6 at 100 µM (lanes 3-8). Samples were then resolved by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were probed with a polyclonal antisera to HGF/SF (NCI-53), and the relative amounts of the HGF proteins were detected by enhanced chemiluminescence (ECL). Figures 7C and 7D are bar graphs depicting the relative amount of pro-HGF/SF observed in Figures 7A and 7B respectively.

Figures 8A, 8B, and 8C show the dependence of inhibition of serum-mediated cleavage of pro-HGF/SF on the concentration of suramin and suramin-related compounds of the present invention. 1 μg of human HGF/SF produced by S114 cells under serum free conditions was incubated overnight in the absence of serum (lanes 1), or in the presence of 5% FBS (lanes 2-8) alone (lanes 2) or in the presence of 5, 50, or 500 μM of the various compounds as indicated (lanes 3-8). Samples were then resolved by SDS-PAGE and transferred to nitrocellulose membrane. The membranes were probed with a polyclonal antisera to HGF/SF (NCI-53), and the relative amounts of the HGF proteins were detected by enhanced chemiluminescence (ECL). Figures 8D, 8E and 8F are bar graphs depicting the relative amount of pro-HGF/SF observed in Figures 8A, 8B and 8C respectively.

Figure 9 depicts the synthesis and structure of an Evans Blue molecule that has been modified by substituting aminoethyl linker groups for the two methyl groups on the biphenyl center. Starting compound I is the biphenyl center with two aminoethyl linker moieties - CH_2CH_2-X in which X is an amine group protected as shown at the top right of Figure 9. Treatment of compound I with $NaNO_2$ in aqueous HCl solution results in formation of compound II, which is then reacted with two substituted naphthylamine compounds to produce compound III, as shown. Deprotection of the amino groups of the aminoethyl linker moieties by treatment with H_2NNH_2 results in compound IV, the modified Evans Blue compound having two aminoethyl linker groups in place of the two methyl groups on the biphenyl center. The amino groups of the aminoethyl linker moieties of Compound IV are readily attached to polyglutamate molecules to yield a polyglutamate-Evans Blue conjugate.

DETAILED DESCRIPTION OF THE INVENTION

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein

can be used in the practice of making and testing of the present invention, the preferred methods and materials are described.

The present inventors have identified a novel activity of suramin and other polysulfonate compounds related to their ability to inhibit the activity of HGF/SF on cells, that is, the ability of these compounds to inhibit the proteolytic cleavage of pro-HGF/SF by serum proteases such as uPA.

S114 cells are highly transformed mouse NIH3T3 fibrobasts which have been engineered to overexpress human HGF/SF and c-Met receptor proteins. These cells rapidly form both tumors and metastases in athymic nude mice. In the presence of serum, the HGF/SF produced by these cells exists predominantly in its cleaved αβ dimeric form (60 and 32-36 kDa respectively, Fig. 1 lane 1). The 32-36 kDa doublet observed at the position of the β chain is due to differences in carbohydrate content (Weidner et al., 1990). However, the inventors observed that a significant proportion of secreted pro-HGF/SF produced by cells growing in the presence of suramin remained uncleaved (90 kDa pro-HGF, lane 3). Heparin, a highly sulfonated protein, had a similar inhibitory effect on pro-HGF/SF cleavage (lane 2).

To begin to address the mechanism through which suramin inhibits cleavage of pro-HGF/SF, an assay was performed in the absence of cells which produce or bind HGF/SF in order to determine whether the inhibition occurs directly at the level of pro-HGF cleavage, or indirectly, perhaps by events occurring at the surface of the HGF/SF-binding cell, e.g., events involving interactions of HGF/SF with heparin sulfate proteoglycan (HSPG)-HGF. In the assay, proteins of S114 cells growing in the absence of serum were labeled with ^{35}S, and the cell supernatant containing ^{35}S-labeled pro-HGF/SF was collected and incubated with serum in the presence and absence of suramin. The resulting ^{35}S-labeled HGF/SF polypeptides were then analyzed. Pro-HGF/SF incubated in the absence of serum is not cleaved (Fig. 2, lane 1); however, as occurs in the presence of the HGF/SF-producing and binding S114 cells, pro-HGF/SF incubated with serum alone is efficiently cleaved (Fig. 2, lane 2), while cleavage of pro-HGF/SF in serum plus suramin (Fig. 2, lane 4) or heparin (Fig. 2 lane 3) is inhibited. The demonstration that suramin inhibits cleavage of pro-HGF/SF in serum in a cell-free assay

suggests that the inhibition might involve binding of suramin to the pro-HGF/SF protein, or to the pro-HGF/SF-cleaving protease.

Serum may contain several different HGF/SF-activating enzymes. Since uPA is one of the molecules which have been shown to activate pro-HGF/SF via cleavage, the effect of suramin on uPA-mediated pro-HGF/SF activation was examined (Fig. 3). Supernatant collected from ^{35}S-labeled S114 cells grown in serum-free media was incubated without uPA (lane 1), with uPA alone (lane 2) or with uPA in the presence of suramin (lane 3). As observed with serum, suramin inhibited uPA-mediated cleavage of pro-HGF/SF in the absence of cells. These results demonstrate that suramin inhibits both serum and uPA-mediated cleavage of pro-HGF/SF.

Administration of suramin as a therapeutic agent is often accompanied by a wide variety of side effects, as discussed above. Suramin is known to complex with serum albumin, and suramin-albumin complexes are readily taken up by cells (Baghdiguian et al., 1991; and Baghdiguian et al., 1996). The toxicity shown by suramin to cells *in vitro* and *in vivo* appears to be due, in part, to intracellular interactions of suramin, e.g., perturbation of the lysosomal system (Baghdiguian et al., 1996) and inhibition of critical cellular enzymes (Cristiani et al., 1995). In order to reduce the cytotoxicity of suramin and suramin-like polysulfonated compounds that inhibit cleavage/activation of HGF/SF, the present invention includes modifying the suramin and suramin-like polysulfonated compounds by attachment of chemical moieties which reduce cellular uptake of the compounds. Examples of such chemical moieties that can be attached to suramin and suramin-like polysulfonated inhibitors of HGF/SF cleavage to inhibit cell-uptake are PEG and polyanionic moieties such as polyglutamate and polyaspartate. The following synthetic method results in attachment of PEG to suramin and suramin-like polysulfonated compounds of the present invention, to reduce their cellular uptake and so limit their cytotoxicity.

The synthesis and structure of a PEG-suramin ester are shown in Fig. 4. Suramin salt is passed through an ion exchange column and the free-acid-containing fraction corresponding to pH<5 is collected and lyophilized. Suramin free acid, PEG-400, and 4-

dimethylaminopyridine (DMAP) are dissolved in dimethylformamid (DMF), and a solution of dicyclohexylcarbodiimid (DCC) in chloroform is added at room temperature. The reaction mixture is stirred at room temperature, e.g. for about 10 hours, filtered, and the solvent is evaporated. The residue is extracted, e.g., with a solution of ethyl ether and chloroform, and the solid residue is dried under reduced pressure. The foregoing method was carried out to produce a product that was 72% PEG-suramin (see Example 4). One skilled in the art would recognize that the number of PEG groups that might be attached to the sulfonic groups of a single suramin molecule can range from zero to six. Analysis of the PEG-suramin product obtained using the method described above under the set of conditions described in Example 4 indicated that the average number of PEG groups attached to each suramin was four. Those skilled in the art would recognize that the conditions of the synthetic reaction can be changed to increase or decrease the average number of PEG groups attached during the reaction. The foregoing method for synthesizing the PEG esters of suramin and other polysulfonate compounds of the present invention will serve to make PEG esters of polysulfonate compounds such as those shown in Figure 6 that inhibit pro-HGF/SF cleavage.

Since cleavage of pro-HGF/SF occurs in the extracellular environment, suramin was modified in order to cause it to be retained in the extracellular milieu. Such a modified compound would display reduced toxicity as a result of its reduced cellular uptake. Accordingly, PEG polymers were chemically attached to the sulfonic groups of suramin to generate a PEG-suramin ester (Fig. 4). The PEG-suramin conjugates inhibited serum-mediated cleavage of pro-HGF, although not as efficiently as does unmodified suramin, as shown in Figure 5. It is within the skill of those in the art to vary the conditions under which PEG is attached to optimize the average number of PEG polymers bound to the modified suramin and suramin-related molecules of the present invention, so as to further improve the inhibitory activity of the PEG-conjugated compounds while retaining the reduced cytotoxicity conferred by attachment of PEG.

Inhibition of uptake of suramin and suramin-like polysulfonated compounds into cells can also be accomplished by attachment of polyanionic moieties such as polyglutamate and

polyaspartate, as stated above. A preferred method for synthesizing a polyglutamate-conjugated Evans Blue compound of the present invention is shown in Figure 9. Aminoethyl or hydroxyethyl linker arms are attached to the compound in the course of synthesis, and these are linked to the polyglutamate or polyaspartate moieties. In practice, several forms of the compound are made which vary in the number of negatively charged glutamate or aspartate subunits per polyanionic moiety, and these are tested to determine the number of subunits to use to give maximal inhibition of cell uptake. Two to ten negatively charged residues per polyglutamate or polyaspartate moiety are usually sufficient to inhibit cell uptake of the HGF/SF cleavage-inhibitng compounds of the present invention.

The inventors of the present invention tested a number of polysulfonate compounds having structures present within, or similar to, those of suramin, to determine whether any of them also possess the ability to inhibit cleavage of pro-HGF/SF. Chemical structures of suramin and the eleven compounds that were investigated are shown in Figure 6. The results of initial screening, using a single 100 μM dose of compound, are shown in Figures 7A and 7B. Suramin, Direct Blue 71, Direct Red 80, Trypan Blue, Direct Red 75 and Evans Blue all displayed a high level of inhibitory activity at the single dose tested. Conversely, no activity was detected for Ponceau S, Direct Yellow 62, Biebrich Scarlet, Xylidine Ponceau 2R and Ponceau SS at the single dose tested. Direct Yellow 50 showed moderate activity. Given that such a large fraction of the polysulfonate compounds shown in Figure 6 showed inhibitory activity when tested at 100 μM, it is possible that many, if not all, of the polysulfonate compounds that did not have detectable activity will successfully inhibit HGF/SF cleavage when tested at higher concentrations.

Compounds displaying the highest inhibitory properties were investigated further over the dose range of 5, 50, and 500 μM (Fig. 8). All of the compounds displayed a dose-dependent inhibition of pro-HGF/SF cleavage, and some, such as Evans Blue and Direct Red 80, retained some inhibitory effects at 5 μM. The observed variation in inhibitory activity among these polysulfonate compounds implies that inhibition is mediated by specific

structure-dependent interactions between the polysulfonate compounds of the present invention and the HGF/SF protein and/or the HGF/SF-cleaving protease.

The present invention includes assay methods which identify compounds that inhibit biological activities of HGF/SF that involve activation of c-Met receptor by the αβ heterodimer form of HGF/SF, based on identifying compounds which inhibit cleavage of HGF/SF by serum proteases such as uPA. It will be appreciated by those skilled in the art that assay of inhibition of HGF/SF cleavage can be carried out in the presence of HGF/SF-producing and/or HGF/SF-binding cells, or in a cell-free solution, in accord with the results described above. It will also be appreciated by the skilled artisan that the HGF/SF-cleaving protease that is added to the assay mixture to detect inhibition of HGF/SF cleavage can be added as a purified enzyme such as uPA, or can be added in impure form, e.g. by adding serum, also in accord with the results described above. In addition, it is well within the skill of those in the art to successfully practice the assay of ability to inhibit HGF/SF cleavage of the present invention using a variety of cell types, reaction buffers and solutions, and reaction conditions, that are different from those used in the examples disclosed herein. The composition of the present invention can comprise a single HGF/SF cleavage-inhibiting compound, or two or more different HGF/SF cleavage-inhibiting compounds in synergistic combination. A preferred range for *in vitro* administration of the compositions of the present invention is from about 0.1 μM to about 20,000 μM; preferably from about 1 μM to about 10,000 μM; more preferably from about 5 μM to about 5,000 μM; and most preferably from about 10 μM to about 1000 μM for the compounds of the present invention.

The present invention also includes methods wherein compounds which inhibit cleavage of HGF/SF as identified by the assay disclosed herein, or compositions containing such compounds, are used to inhibit cleavage of HGF/SF in cell-free solutions, in solutions containing cells cultured *in vitro*, and in extracellular solutions *in vivo*. Such methods are useful for preventing cleavage of pro-HGF/SF during storage or study of the protein, in studies of the biochemical pathways and cellular responses involved in HGF/SF activation of

c-Met receptor, and in studies of the role of HGF/SF in promoting angiogenesis, tumor vascularization, tumorigenesis, and tumor metastasis *in vivo*.

It remains to be demonstrated that the ability of suramin to inhibit activation of pro-HGF/SF contributes to its observed anti-tumor activities *in vivo*; however, considerable evidence exists that indicates that this is the case. Aberrations in Met-HGF/SF signaling have been shown to contribute to both tumor growth and metastasis in a variety of systems (see Jeffers et al., 1996a). In addition, it has been observed that some of the cancers in which suramin has shown some therapeutic promise, notably prostate cancer, have also been associated with aberrations in Met-HGF/SF expression/signaling (Humphrey et al., 1995). Suramin is known to inhibit the binding of a variety of growth factors to their receptors (Stein, 1993). In this regard, suramin was shown to inhibit HGF/SF induced scattering of MDCK cells (Adams et al., 1991). It was assumed that this was due entirely to inhibition of Met-HGF binding. Suramin is also reported to induce the "shedding" of Met from the cell surface, reducing the amount of cellular Met receptor, and resulting in the release of a soluble extracellular portion of Met into the extracellular environment, where it may act to sequester free ligand (Galvani et al., 1995). In view of the discoveries of the present invention, it is the inventors' belief that the relative success of suramin treatment in patients with cancer that is associated with aberrations in Met-HGF/SF expression/signaling is due, at least in part, to the ability of suramin to inhibit serum-mediated cleavage and activation of HGF/SF as described in this specification.

Accordingly, the present invention includes pharmaceutical compositions comprising compounds other than suramin which inhibit cleavage of HGF/SF as identified by the assay of the present invention, and methods wherein such pharmaceutical compositions are administered to a mammal with a HGF/SF-stimulated tumor, in order to inhibit the growth or metastasis of the tumor.

The present invention further includes attaching to suramin, and to other polysulfonated compounds which inhibit serum-mediated HGF/SF cleavage, chemical groups that reduce cellular uptake of the compounds and hence reduce their cytotoxicity. Among the

chemical groups which can be attached to suramin and other polysulfonated compounds according to the present invention to reduce cytotoxicity are PEG and polyanionic polymers such as polyglutamate and polyaspartate. PEG polymers can be attached directly to the sulfonic groups of suramin and other polysulfonated compounds of the present invention to produce modified forms of the compounds which retain potent activity as inhibitors of HGF/SF cleavage (Examples 4 & 5). Alternatively, linker moieties such as hydroxyethyl- or aminoethyl- groups can be added to appropriate sites of the HGF/SF cleavage-inhibiting compounds, such as the methyl moieties of Evans Blue or Trypan Blue, and polyanionic polymers such as polyglutamate or polyaspartate can be attached to the reactive linker groups, to produce modified forms of said compounds which also inhibit cleavage of HGF/SF (Example 8). The modified compounds of the present invention are not taken up into cells as efficiently as the unmodified compounds, and so are less cytotoxic. Furthermore, the modified compounds of the present invention remain in the extracellular environment where cleavage of pro-HGF/SF occurs, and so have a longer inhibitory half-life *in vivo*.

As the modified suramin and other polysulfonate compounds of the present invention inhibit serum-mediated cleavage of HGF/SF, as shown by the disclosed assays of the present invention, they retain activity as inhibitors of the HGF/SF:Met signaling pathway, and so possess anti-tumor activity. Accordingly, the present invention further provides suramin and other polysulfonated compounds that are chemically modified so as to have reduced cell-penetrating properties and reduced cytotoxicity, which compounds inhibit tumor vascularization, growth, and metastasis. The present invention further provides pharmaceutical compositions containing such compounds, and methods in which such compositions are provided to a mammal with a tumor that is stimulated to grow by HGF/SF, in order to inhibit the growth of the tumor.

The pharmaceutical compositions of the present invention can comprise a single HGF/SF cleavage-inhibiting compound or two or more different such compounds, and may further include other types of anti-tumor agents, in synergistic combination. The pharmaceutical compositions of the present invention can be administered either parenterally

or non-parenterally, by means that are commonly used by those skilled in the art to administer small, hydrophilic, therapeutic agents. Such means include, but are not limited to, intravenous, intraperitoneal, oral, rectal, ocular, and respiratory routes of administration. The compositions of the present invention are administered in substantially non-toxic dosage concentrations sufficient to insure the release of a sufficient dosage unit of at least one HGF/SF cleavage-inhibiting compound of the present invention into the patient to provide the desired inhibition of tumor growth or metastasis. For the compositions comprising a synergistic combination, these are also administered in substantially non-toxic dosage concentrations sufficient to insure the release of a sufficient dosage unit of the synergistic combination into the patient to provide the desired inhibition of tumor growth or metastasis. The pharmaceutical compositions of the present invention comprising one or more of the herein disclosed HGF/SF cleavage-inhibiting compounds, such as for example, PEG-suramin, Trypan Blue, or polyglutamate-Evans Blue, may contain these compounds in a concentration range of from about 0.1 μM to about 20,000 μM; preferably from about 1 μM to about 10,000 μM; more preferably from about 5 μM to about 5,000 μM; and most preferably from about 10 μM to about 1,000 μM. The actual dosage administered will be determined by physical and physiological factors such as age, body weight, severity of condition, and/or clinical history of the patient. With these considerations in mind, the dosage of the compositions comprising an additive or synergistic combination for a particular subject can be readily determined by the physician. It might be noted that in extreme cases a dosage approaching the toxic level may be the acceptable treatment protocol.

The following examples further demonstrate several preferred embodiments of this invention. Those skilled in the art will recognize numerous equivalents to the specific embodiments described herein. Such equivalents are intended to be within the scope of the claims.

EXAMPLE 1

Inhibition of serum-mediated cleavage pro-HGF/SF by suramin and heparin in the presence of cells that produce and bind HGF/SF.

Proteins of S114 cells were metabolically labeled with ^{35}S overnight in the presence of 5% serum alone, or in serum plus 500 μg/ml heparin or 100 μg/ml (~70 μM) suramin. Cell culture supernatants were then collected, and secreted HGF/SF polypeptides were immunoprecipitated using a polyclonal anti-HGF/SF antisera (clone NCI-53). Resulting immune complexes were then separated by SDS-PAGE, and the different ^{35}S-labeled HGF/SF polypeptides were detected by autoradiography. Figure 1 shows the immunoprecipitated ^{35}S-labeled HGF/SF polypeptides prepared by the above-described method after incubating the S114 cells in the presence of 5% serum alone (lane 1), in serum plus 500 μg/ml heparin (lane 2), or in serum plus 100 μg/ml (~70 μM) suramin (lane 3).

EXAMPLE 2

Inhibition of serum-mediated pro-HGF/SF cleavage by suramin and heparin in cell-free solution.

Proteins of S114 cells were metabolically labeled with ^{35}S overnight in the absence of serum. The tissue culture supernatant was then collected and incubated overnight in the absence of serum, or in the presence of 5% fetal bovine serum (FBS) either alone, or in serum plus 500 μg/ml heparin or 100 μg/ml (~70 μM) suramin. HGF/SF polypeptides were then immunoprecipitated using a polyclonal anti-HGF/SF antisera (clone NCI-53). Resulting immune complexes were then separated by SDS-PAGE, and the different ^{35}S-labeled HGF/SF polypeptides were detected by autoradiography. Figure 2 shows the immunoprecipitated ^{35}S-labeled HGF/SF polypeptides prepared by the above-described method after incubating the cell supernatant in the absence of serum (lane 1), or in the presence of 5% FBS (lanes 2-4), either alone (lane 2) or in the presence of 500 μg/ml heparin (lane 3) or 100 μg/ml (~70 μM) suramin (lane 4).

EXAMPLE 3

Inhibition of urokinase (uPA)-mediated pro-HGF/SF cleavage by suramin and heparin in cell-free solution.

Proteins of S114 cells were metabolically labeled with ^{35}S overnight in the absence of serum. The tissue culture supernatant was then collected and incubated overnight in the absence of uPA, or in the presence of 2 μg (~160 IU) of high molecular weight uPA, either alone or in the presence of 100 μg/ml (~70 μM) suramin. HGF/SF polypeptides were then immunoprecipitated using a polyclonal anti-HGF/SF antisera (clone NCI-53). Resulting immune complexes were then separated by SDS-PAGE, and the different ^{35}S-labeled HGF/SF polypeptides were detected by autoradiography. Figure 3 shows the immunoprecipitated ^{35}S-labeled HGF/SF polypeptides prepared by the above-described method after incubating the cell supernatant in the absence (lane 1) or presence of 2 μg (~160 IU) of high molecular weight uPA (lanes 2 & 3), either alone (lane 2) or in the presence of 100 μg/ml (~70 μM) suramin (lane 3).

EXAMPLE 4

Synthesis of PEG-suramin esters.

Suramin salt (Mobay Chemical Corp., New York) was passed through an ion exchange (Dowex) column, and the fraction corresponding to pH < 5 was collected and lyophilized. Suramin free acid (130 mg; 0.1 mmole), PEG-400 (280 mg; 0.7 mmole) and 4-dimethylaminopyridine (DMAP) (5 mg) were dissolved in dimethylformamide (DMF)(3 ml). A solution of dicyclohexylcarbo-diimid (DCC)(147 mg; 0.7 mmole) in chloroform (1 ml) was added at room temperature, and the reaction mixture was then stirred at room temperature for 10 hours. The precipitate of urea was filtered off and the solvent was evaporated under reduced pressure. The residue was extracted with a 2:1 mixture of ethyl ether and chloroform (2 x 10 ml). The solid residue was dried under reduced pressure. Yield: 210 mg (72%).

NMR analysis: NMR (DMSO-d_6) chemical shift (multiplicity, coupling constant, number of protons):10.07 (s,2H), 9.38 (d,1.9, 2H), 8.96 (s,2H), 8.60 (d,1.9,2H), 8.23 (s,4H), 8.06 (d,8.2,2H), 8.05 (dd,7.8,1.8,2H), 8.03 (d,8.2,2H), 8.00 (d,1.7,2H), 7.93 (dd,1.7,1.0,2H), 7.87 (ddd,7.5,1.8,0.8,2H), 7.65 (ddd,8.0,1.6,0.8,2H), 7.46 (dd,7.8,7.8,2H), 7.39 (d,8.2,2H), 3.65-3.47 (m,112H), 3.47-3.39 (m, overlapped with H_2O,16H), 2.33(s,6H).

It is estimated that the resulting suramin-PEG esters contained, on average, approximately four PEG moieties per molecule.

EXAMPLE 5

Inhibition of serum-mediated pro-HGF/SF cleavage by PEG-suramin esters in cell-free solution.

Proteins of S114 cells were metabolically labeled with ^{35}S overnight in the absence of serum. The tissue culture supernatant was then collected and incubated overnight in the absence of serum, or in the presence of 5% serum, either alone or in the presence of 200 µg/ml PEG-suramin. HGF/SF polypeptides were then immunoprecipitated using a polyclonal anti-HGF/SF antisera (clone NCI-53). Resulting immune complexes were then separated by SDS-PAGE, and the different ^{35}S-labeled HGF/SF polypeptides were detected by autoradiography. Figure 5 shows the immunoprecipitated ^{35}S-labeled HGF/SF polypeptides prepared by the above-described method after incubating the cell in the absence (lane 1) or presence of 5% serum (lanes 2 & 3), either alone (lane 2) or in the presence of 200 µg/ml PEG-suramin (lane 3).

EXAMPLE 6

Inhibition of serum-mediated pro-HGF/SF cleavage by suramin-related and polysulfonated compounds.

Human HGF/SF was purified from S114 cells under serum-free conditions. 1 µg of HGF/SF was then incubated overnight in the absence of serum, or in the presence of 5% FBS, alone or in the presence of 100 µM of each of the compounds shown in Figure 6. Samples

were then resolved by SDS-PAGE and transferred to nitrocellulose membrane. Blots were then probed with a polyclonal antisera to HGF/SF (NCI-53), and the relative amounts of the different HGF/SF polypeptides were detected by enhanced chemiluminescence (ECL). Figures 7A and 7B show the relative amounts of the different HGF/SF polypeptides produced by incubating HGF/SF in the absence of serum (lanes 1), or in 5% FBS alone (lanes 2) or plus each of the compounds shown in Figure 6 (100 μM) (lanes 3-8, as indicated). Figures 7C and 7D are bar graphs depicting the relative amount of pro-HGF/SF observed in Figures 7A and 7B respectively.

EXAMPLE 7

Concentration-dependence of inhibition of serum-mediated pro-HGF/SF cleavage by polysulfonated compounds.

Human HGF/SF was purified from S114 cells under serum-free conditions. 1 μg of HGF/SF was then incubated overnight in the absence of serum, or in the presence of 5% FBS alone, or in the presence of 5, 50, or 500 μM of selected compounds shown in Figure 6. Samples were then resolved by SDS-PAGE, and transferred to nitrocellulose membrane. Blots were then probed with a polyclonal antisera to HGF (clone NCI-53), and the relative amounts of the different HGF/SF polypeptides were detected by enhanced chemiluminescence (ECL). Figures 8A, 8B, and 8C show the relative amounts of the different HGF/SF polypeptides produced by incubating HGF/SF in the absence of serum (lanes 1), or in 5% FBS alone (lanes 2) or in the presence of the selected compounds at 5, 50, and 500 μM as indicated (lanes 3-8). Figures 8D, 8E and 8F are bar graphs depicting the relative amount of pro-HGF/SF observed in Figures 8A, 8B and 8C respectively.

EXAMPLE 8

Synthesis of polyglutamate-Evans Blue conjugate.

Polyglutamate-Evans Blue conjugate is produced by reacting polyglutamate polymers with a modified Evans Blue compound having two aminoethyl linker groups in place of the

two methyl groups on the biphenyl center (compound IV in Figure 9). The reaction sequence for synthesizing the modified Evans Blue compound having the two aminoethyl linker groups is shown in Figure 9. Compound I is a substituted biphenyl starting compound with two aminoethyl linker moieties $-CH_2CH_2-X$ in which X is an amine group protected as shown at the top right of Figure 9. Treating compound I with $NaNO_2$ in aqueous HCl solution results in formation of compound II. Compound II reacts with the two substituted naphthylamine compounds to produce compound III, as shown in Figure 9. Deprotection of the amino groups of the aminoethyl linker moieties by treatment with H_2NNH_2 results in compound IV, the modified Evans Blue compound having two aminoethyl linker groups in place of the two methyl groups on the biphenyl center. The amino groups of the linkers of Compound IV are readily attached to polyglutamate molecules to yield a polyglutamate-Evans Blue conjugate.

An alternative method for making a polyglutamate-Evans Blue conjugate is to start with compound I having two hydroxyethyl linker groups $-CH_2CH_2-X$, in which X is a hydroxyl group that is protected by a suitable protecting moiety, and to proceed with the steps of the synthesis as described above for the compound having aminoethyl linker groups. Deprotection then gives a compound similar to compound IV but with two hydroxyethyl instead of aminoethyl linker groups (Y is -OH). The hydroxyl groups of the linkers of this compound are also readily linked to polyglutamate to yield a polyglutamate-Evans Blue conjugate. The synthetic reactions shown in Figure 9 and described above involve reactions and reaction conditions that are known to those skilled in the art of organic chemical synthesis.

It will be understood that certain features and subcombinations of the disclosed invention are of utility and may be employed without reference to other features and subcombinations. This is contemplated by and is within the scope of the claims. Since many possible embodiments may be made of the invention without departing from the scope thereof, it is to be understood that all matter herein set forth or shown in the accompanying drawings and figures is to be interpreted as illustrative and not in a limiting sense. All references cited are specifically incorporated herein by reference.

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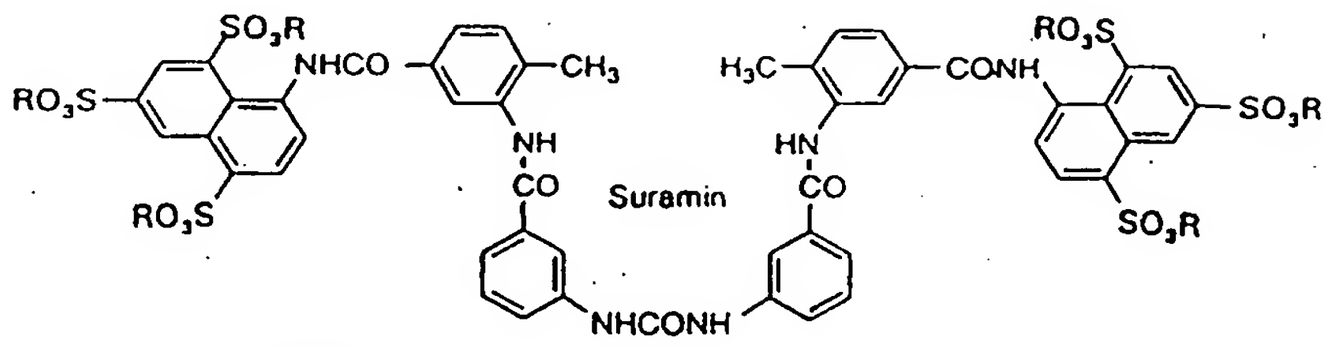
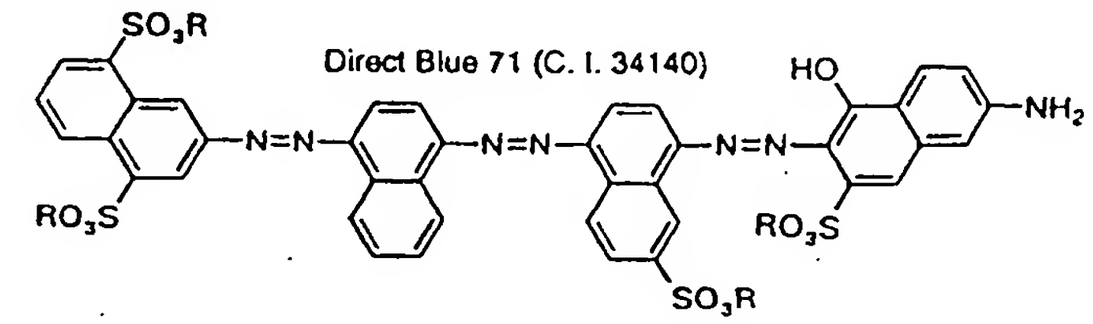
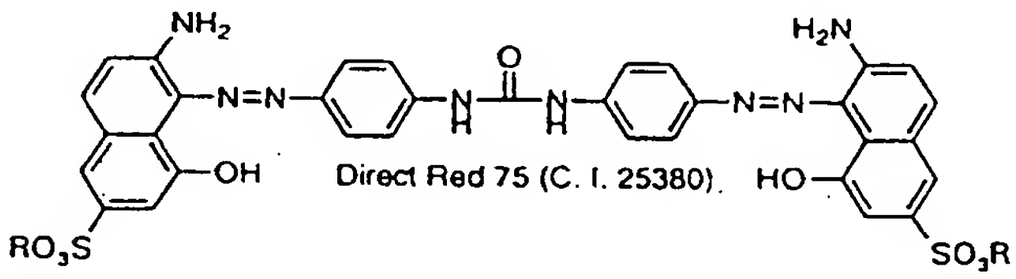
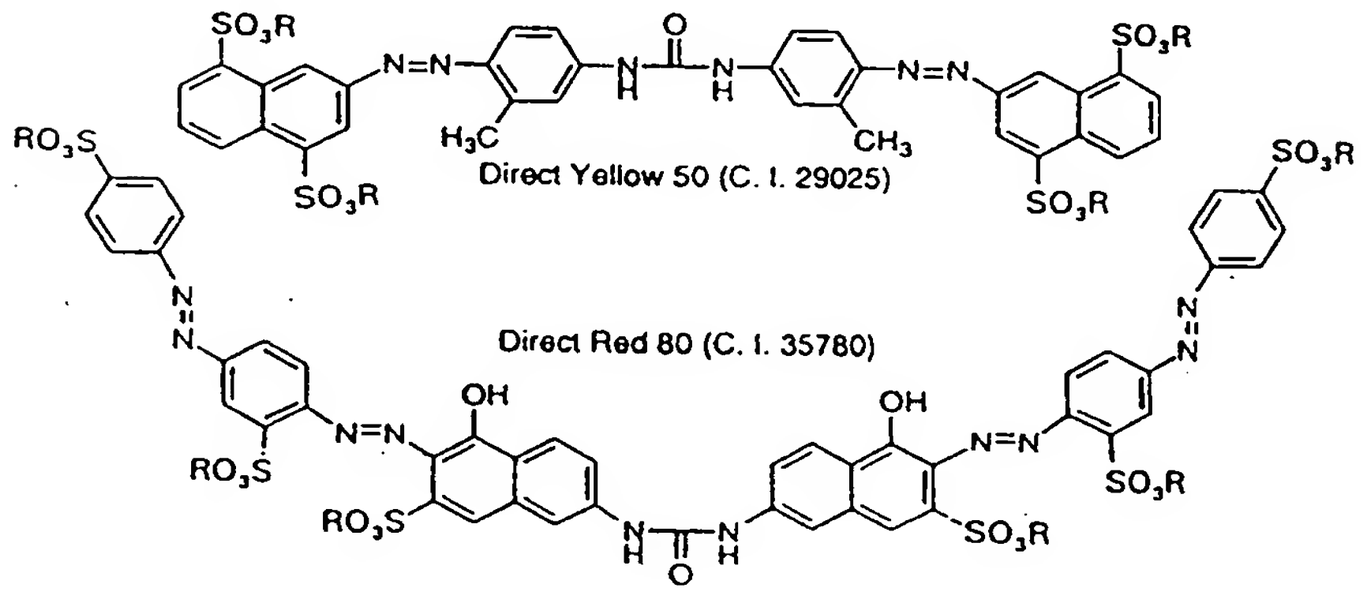
WHAT IS CLAIMED IS:

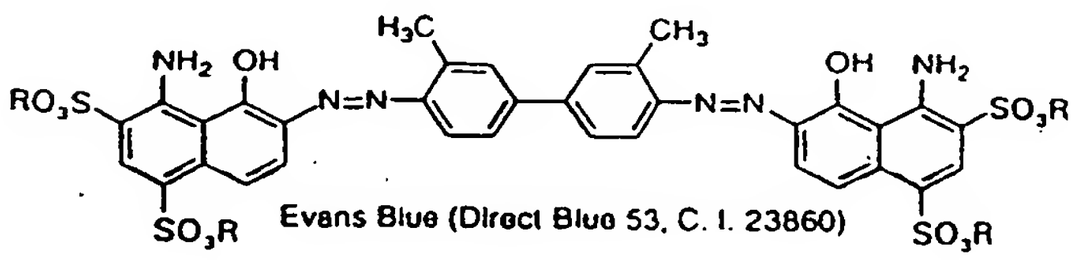
1. A compound selected from the group consisting of:

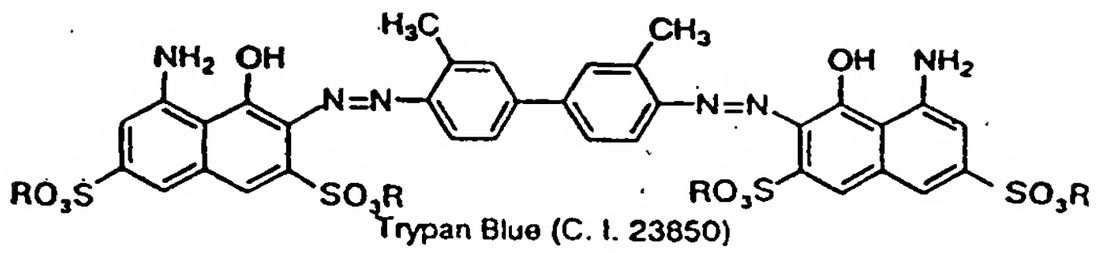












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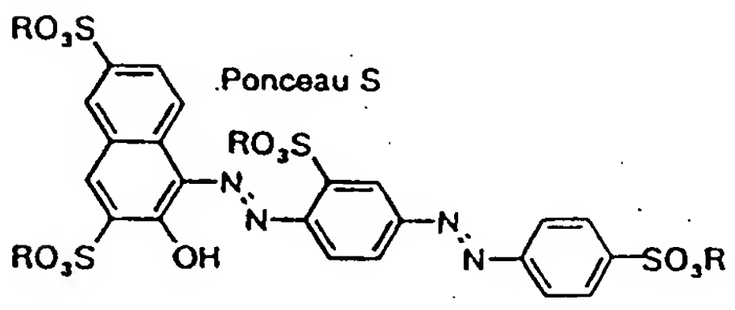

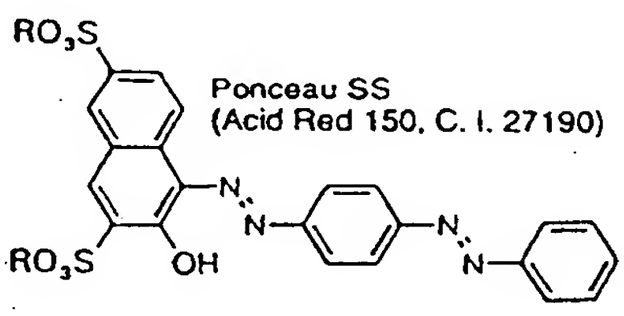

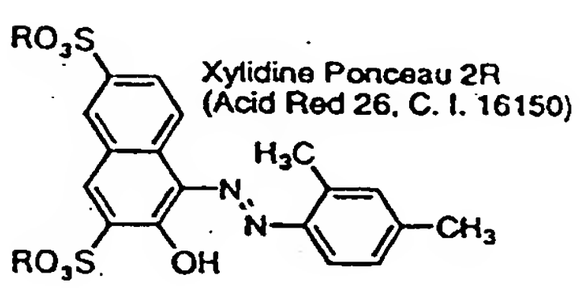

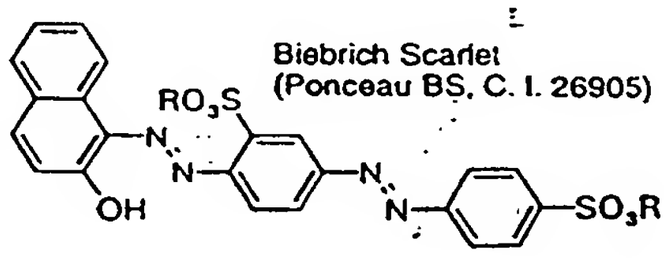

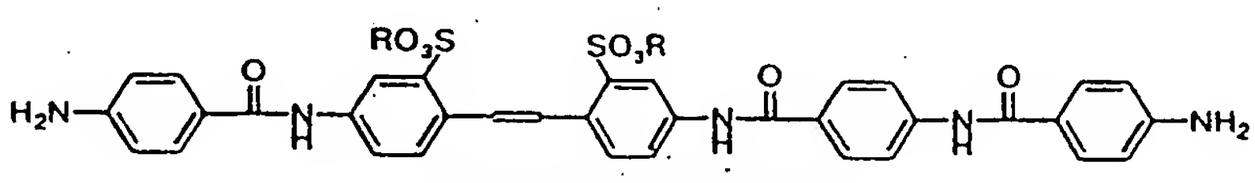

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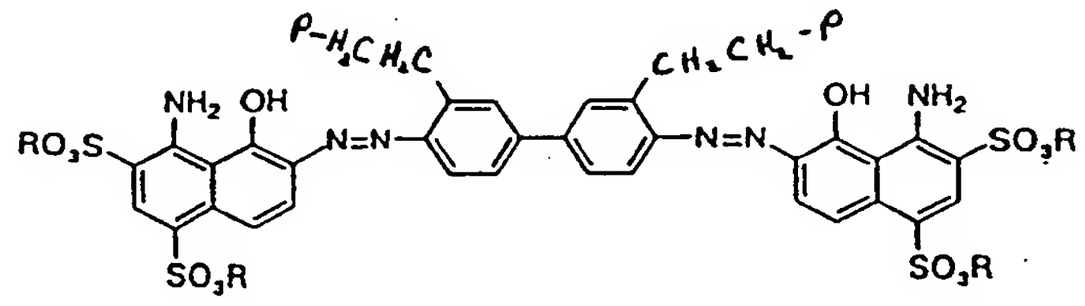
wherein each R is independently selected from the group consisting of PEG, H, and a cationic counterion, and at least one R moiety of the compound is PEG, or

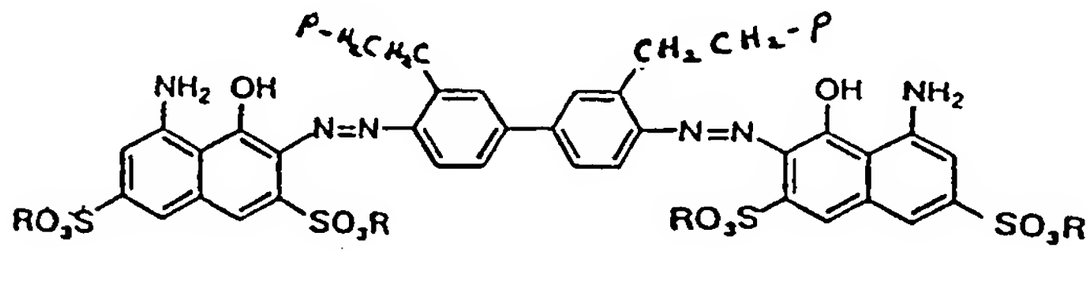
wherein each R is independently selected from the group consisting of H and a cationic counterion, and the compound is conjugated to at least one polyanionic moiety.

2. The compound of claim 1 wherein wherein each R is independently selected from the group consisting of H and a cationic counterion, and the compound is conjugated to at least one polyaspartate or polyglutamate moiety.

3. The compound of claim 2 selected from the group consisting of:



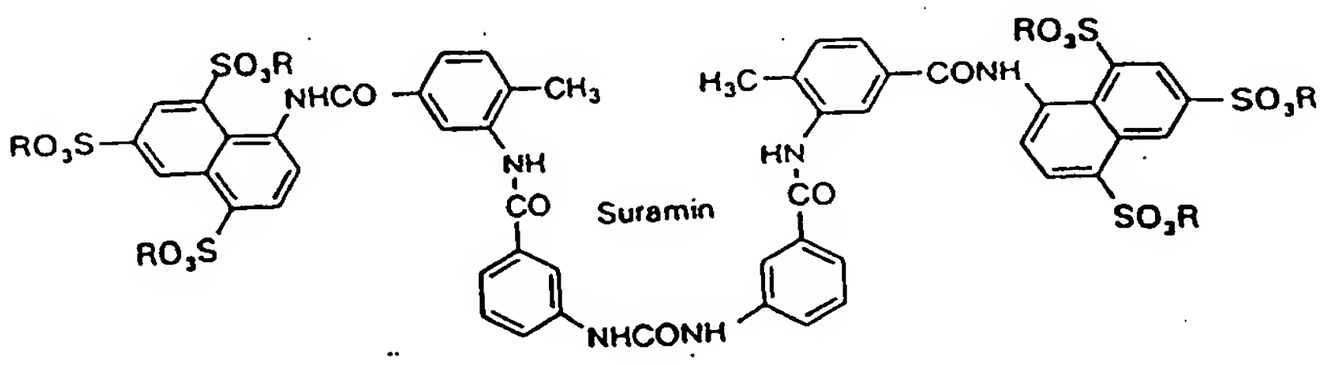
and

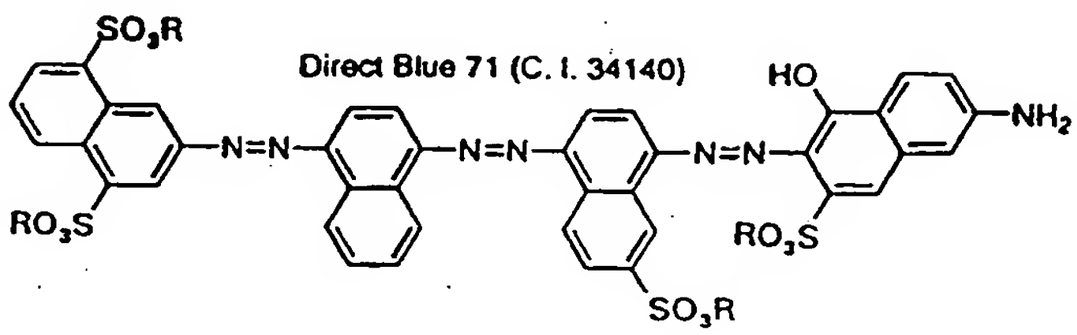


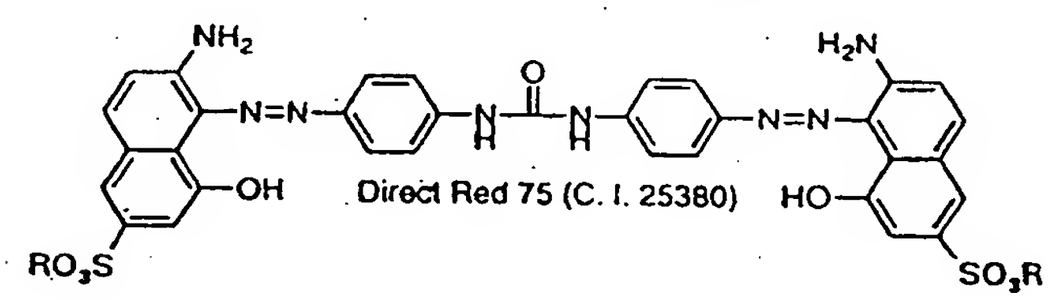
wherein both P moieties of the compound are the same and are selected from the group consisting of: -O-polyglutamate, -NH-polyglutamate, -O-polyaspartate, and -NH-polyaspartate.

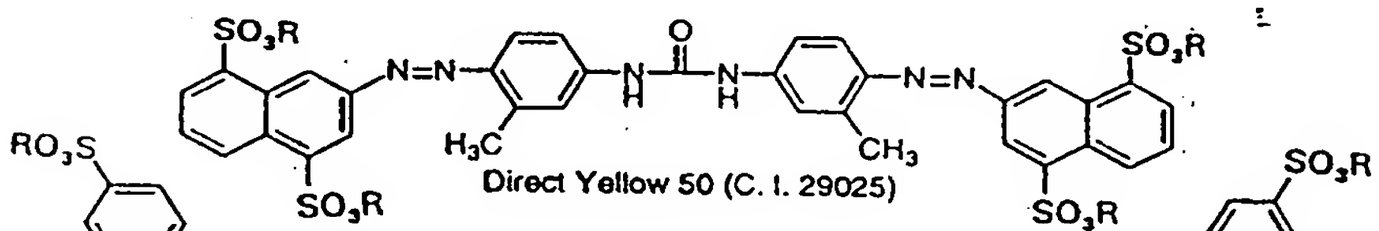
4. A method of inhibiting cleavage of HGF/SF in a solution by a protease, the method comprising:

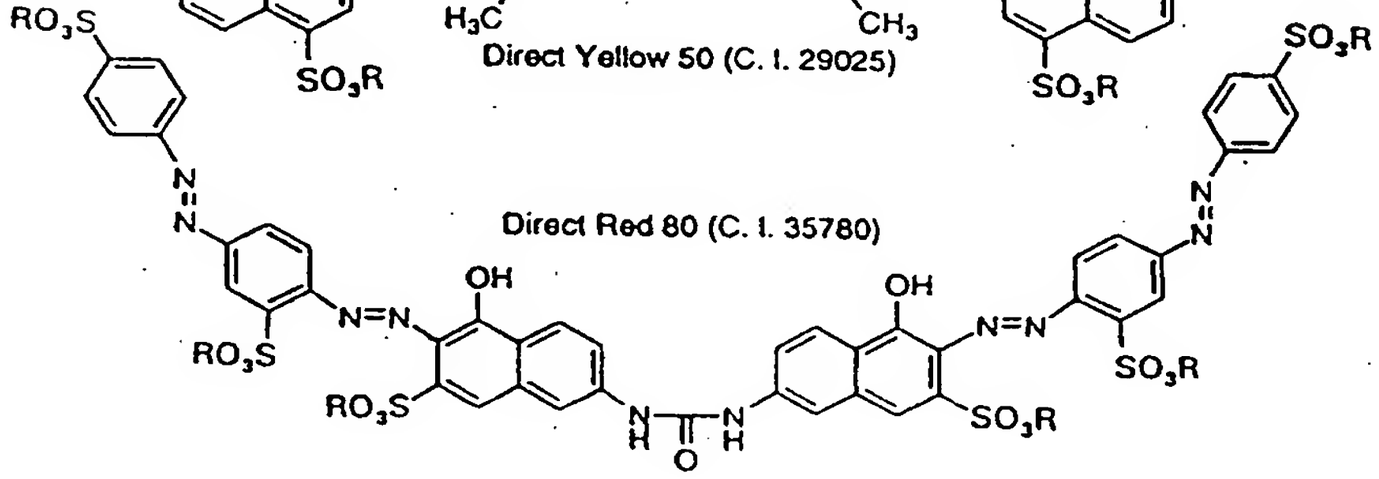
providing to a solution comprising intact HGF/SF, or providing to a solution comprising the protease which is to be mixed with a solution comprising intact HGF/SF, a compound selected from the group consisting of:

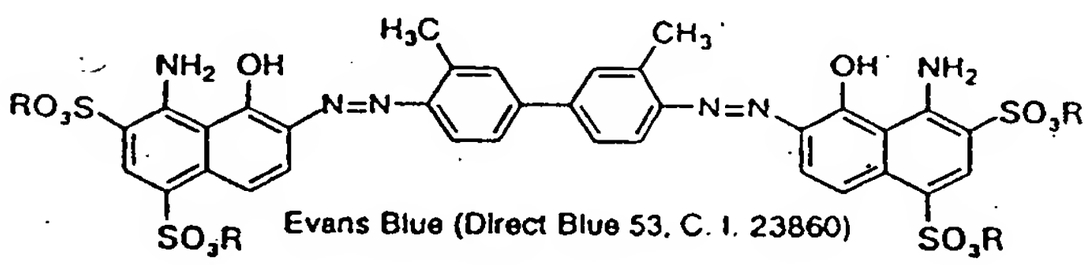


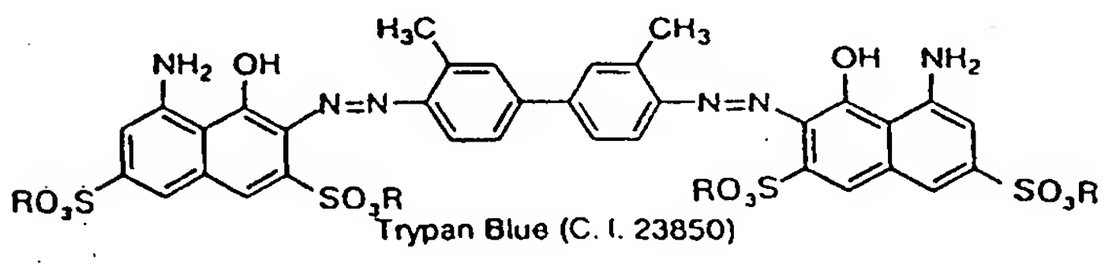


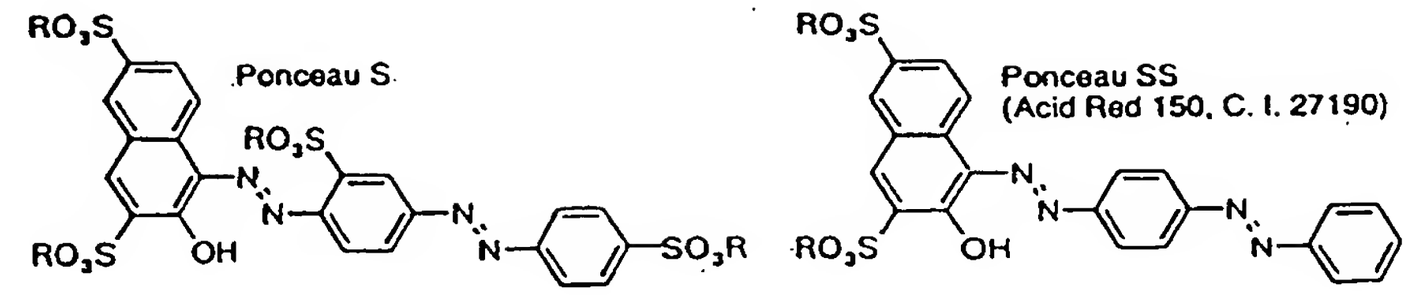


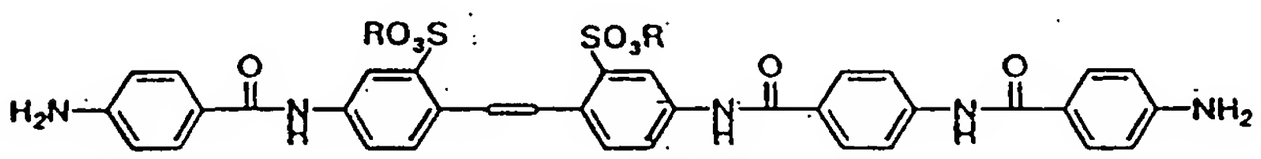












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wherein each R moiety is independently selected from the group consisting of PEG, H, and a cationic counterion, or

wherein each R is independently selected from the group consisting of H and a cationic counterion, and the compound is conjugated to at least one polyanionic moiety.

5. The method of claim 4 wherein cleavage of HGF/SF by the uninhibited protease produces an α peptide of about 60 kDa and a β peptide of about 32-36 kDa as cleavage products.

6. The method of claim 4 wherein the protease which is inhibited from cleaving HGF/SF is a serum protease.

7. The method of claim 4 wherein the protease which is inhibited from cleaving HGF/SF is urokinase.

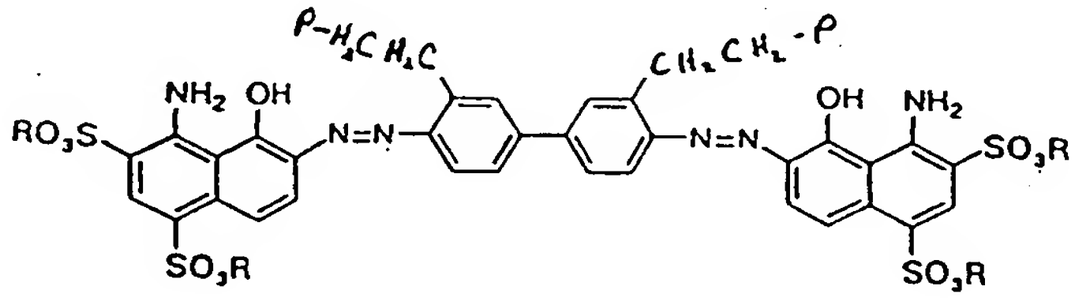
8. The method of claim 4 wherein the compound that inhibits cleavage of HGF/SF is provided to a solution *in vitro*.

9. The method of claim 4 wherein the compound that inhibits cleavage of HGF/SF is provided to an extracellular solution of a mammal *in vivo* to inhibit cleavage of HGF/SF present in said extracellular solution.

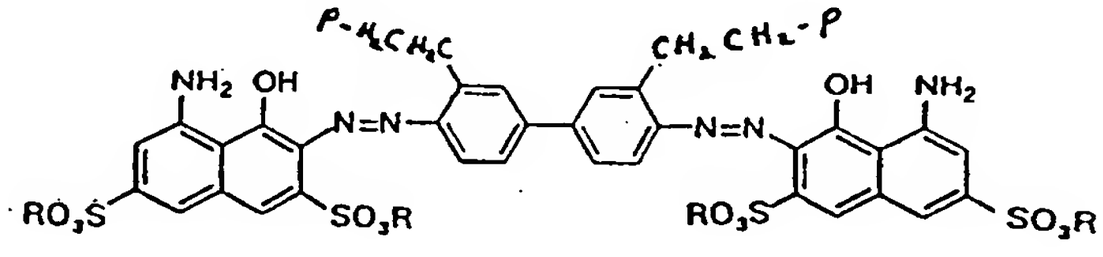
10. The method of claim 4 wherein at least one R moiety of the compound is PEG.

11. The method of claim 4 wherein the compound is conjugated to at least one polyaspartate or polyglutamate moiety.

12. The method of claim 4 wherein the compound is selected from the group consisting of:



and



wherein both P moieties of the compound are the same and are selected from the group consisting of: -O-polyglutamate, -NH-polyglutamate, -O-polyaspartate, and -NH-polyaspartate.

13. A composition comprising at least one compound according to claim 1, and further comprising a pharmaceutically acceptable carrier.

14. A method of inhibiting tumor growth in a mammal comprising administering to a mammal in need of such treatment an effective amount of a composition according to claim 13.

15. A method of inhibiting tumor metastasis in a mammal comprising administering to a mammal in need of such treatment an effective amount of a composition according to claim 13.

16. A method for making a compound according to claim 1 wherein at least one R moiety is PEG, comprising:

dissolving in dimethylformamid: (i) a compound according to claim 1 wherein R is H, (ii) PEG, and (iii) 4-dimethylaminopyridine

adding a solution of dicyclohexylcarbidiimid in chloroform,

and stirring until the desired compound is formed,

filtering to remove precipitate,

evaporating off the solvent,

extracting the residue with organic solvent, and

drying the resulting solid residue, which contains the desired compound.

17. A method for identifying a compound that inhibits cellular responses induced by activation of c-Met receptor by HGF/SF, the method comprising:

providing the compound to a solution comprising intact HGF/SF, or to a solution comprising a protease which cleaves HGF/SF in its uninhibited state to produce an α peptide of about 60 kDa and a β peptide of about 32-36 kDa,

adding the solution comprising intact HGF/SF to the solution comprising the protease which cleaves HGF/SF in its uninhibited state,

incubating the combined solutions, and

analyzing the HGF/SF polypeptides present in the reaction mixture to determine if the compound inhibited cleavage of HGF/SF by the protease,

wherein inhibition of cleavage of HGF/SF indicates that the compound being tested inhibits cellular responses induced by activation of c-Met receptor by HGF/SF.

18. The method of claim 17 wherein the protease which cleaves HGF/SF in its uninhibited state is a serum protease.

19. The method of claim 17 wherein the protease which cleaves HGF/SF in its uninhibited state is urokinase.

20. The method of claim 17 wherein the compound to be tested and the solution comprising intact HGF/SF are added to a solution comprising the protease *in vitro*.

21. The method of claim 17 wherein the compound to be tested is added to an extracellular solution of a mammal *in vivo*.

22. The method of claim 17 wherein the cellular responses induced by activation of c-Met receptor by HGF/SF are selected from the group consisting of angiogenesis, mitogenesis, morphogenesis, cell scattering, tumor vascularization, tumor growth, and tumor metastasis.

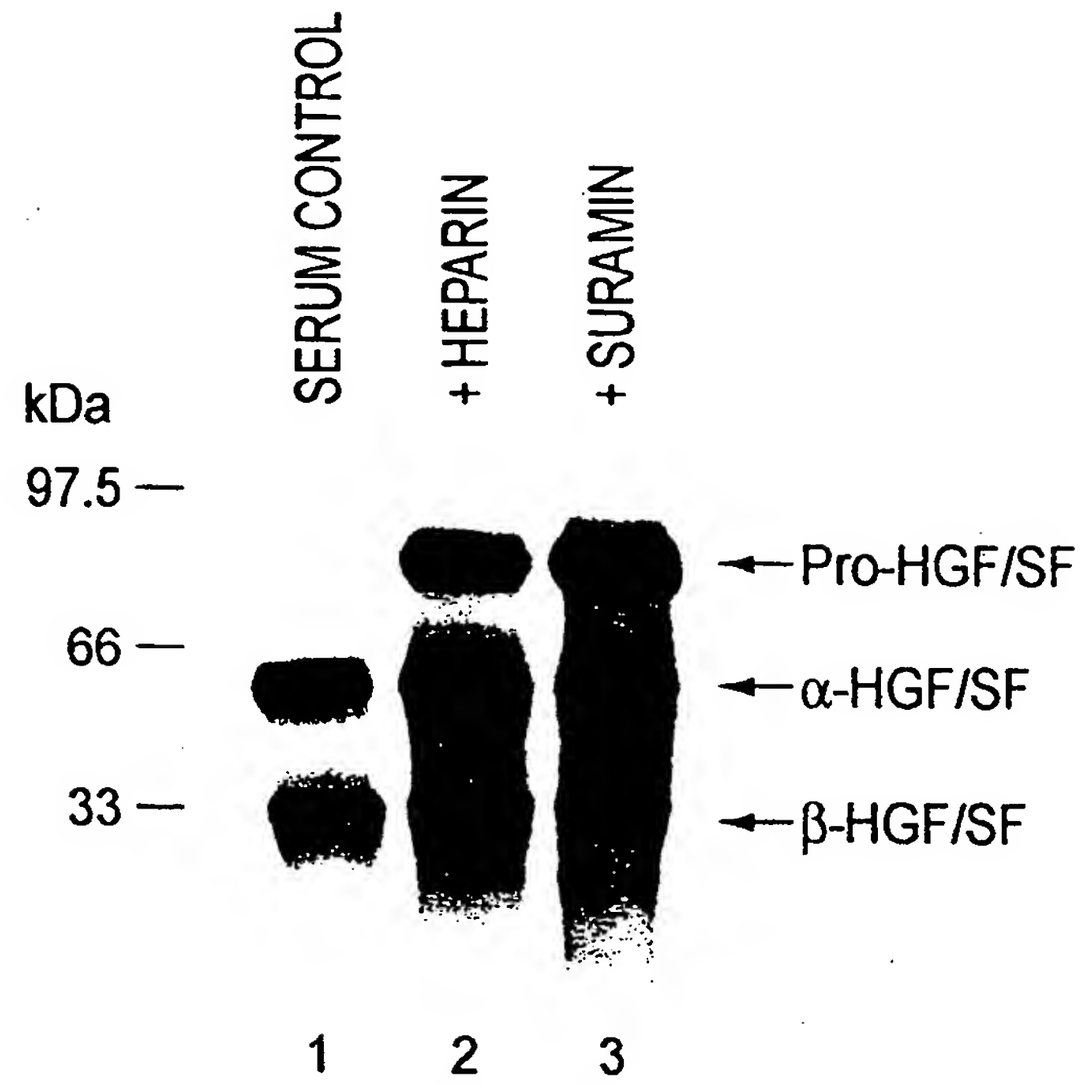


FIG. 1

SERUM	−	+	+	+
SURAMIN	−	−	−	+
HEPARIN	−	−	+	−

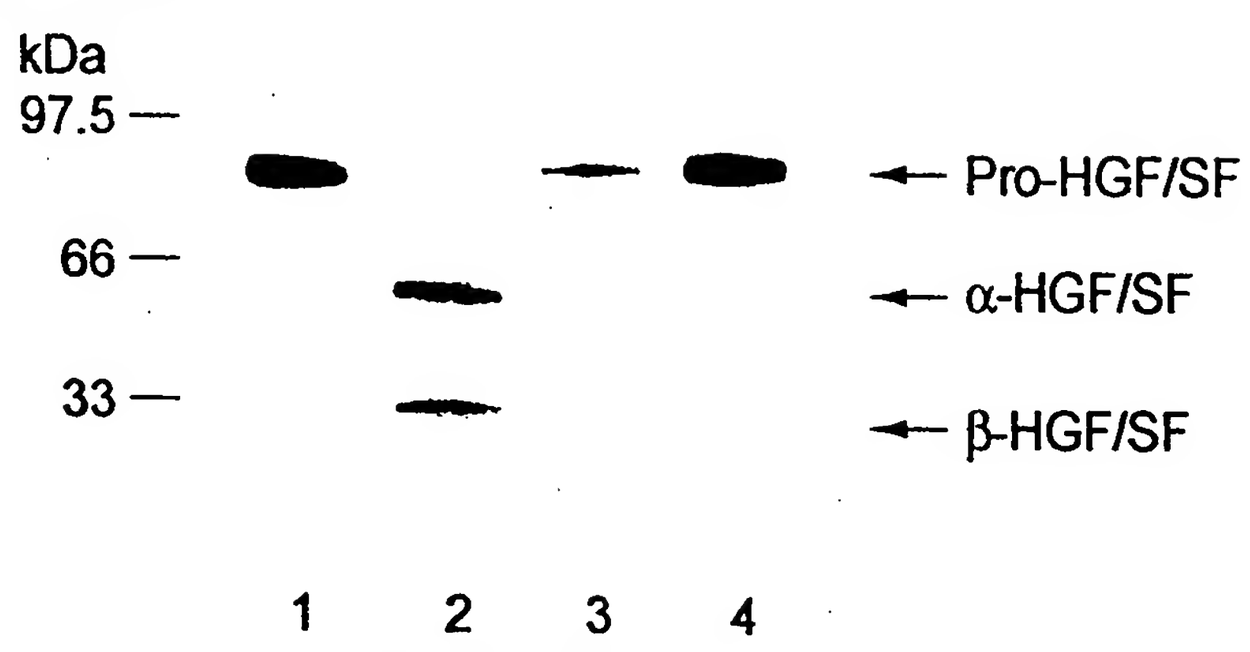


FIG. 2

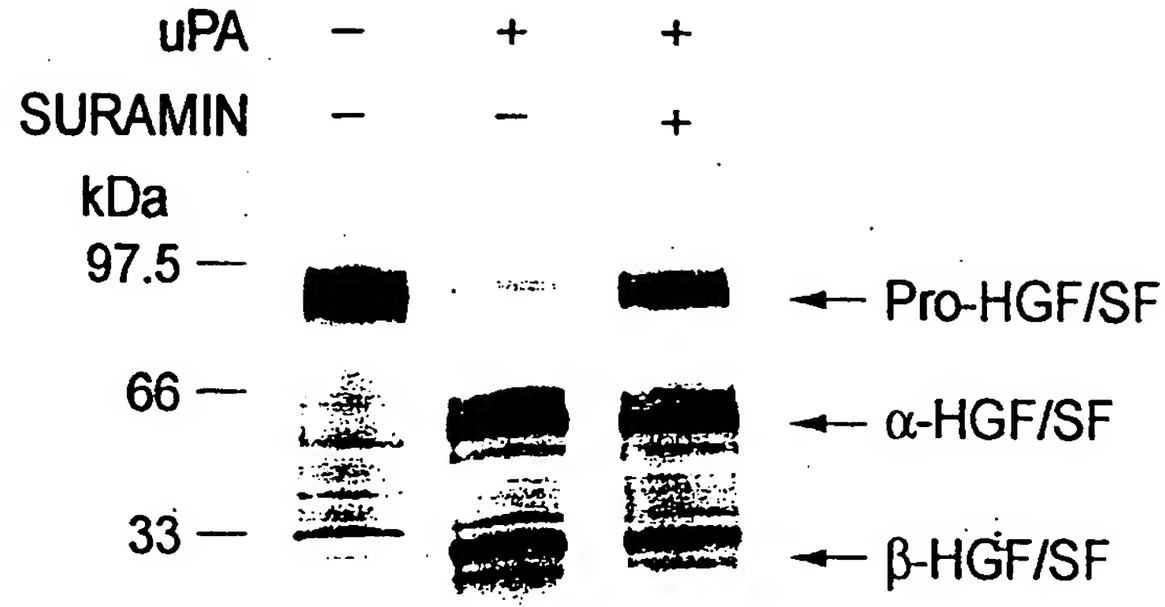

uPA – + +
SURAMIN – – +
kDa
97.5
66
33
Pro-HGF/SF
α-HGF/SF
β-HGF/SF
1 2 3

FIG. 3

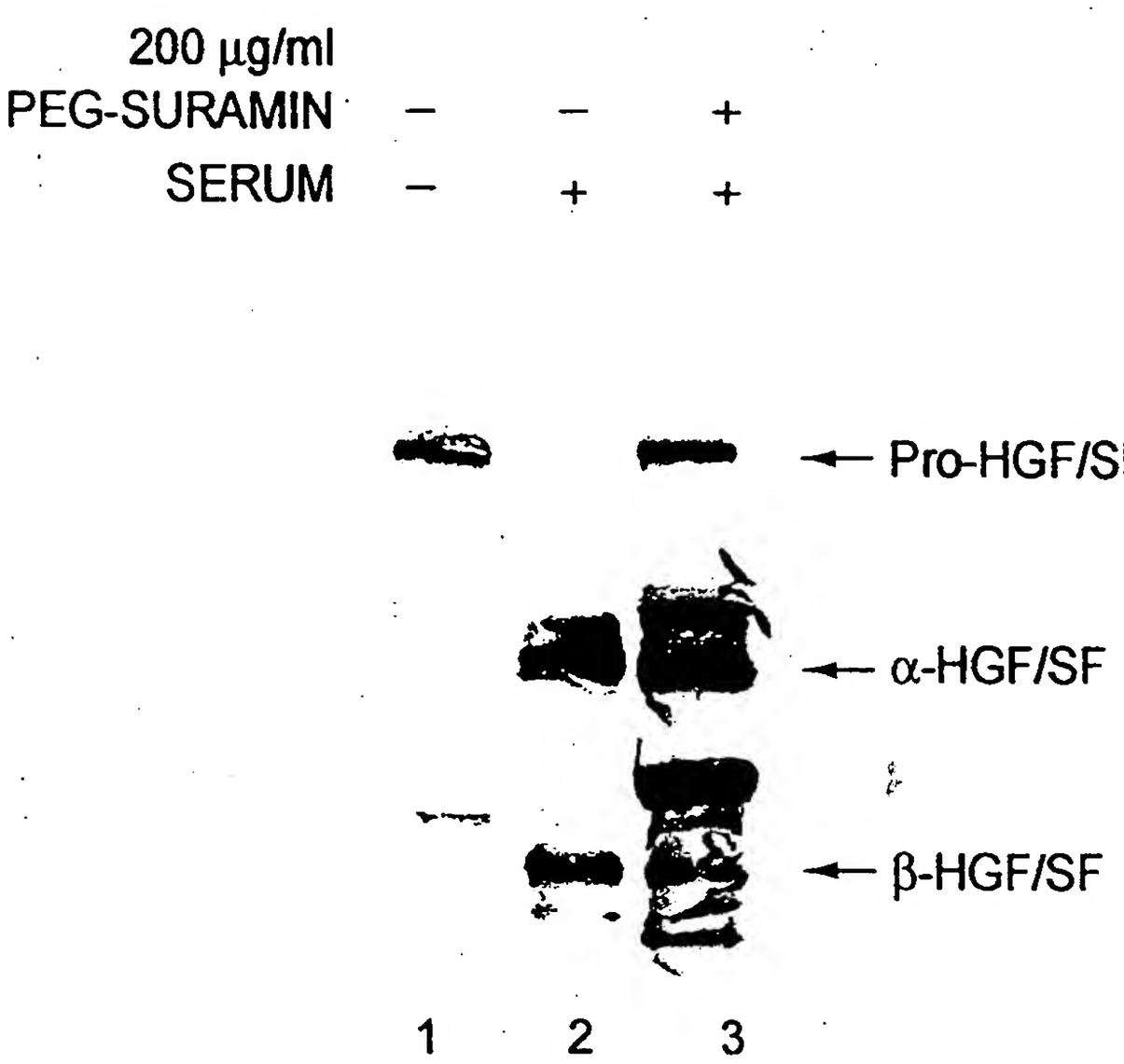

200 µg/ml
PEG-SURAMIN – – +
SERUM – + +
Pro-HGF/SF
α-HGF/SF
β-HGF/SF
1 2 3

FIG. 5

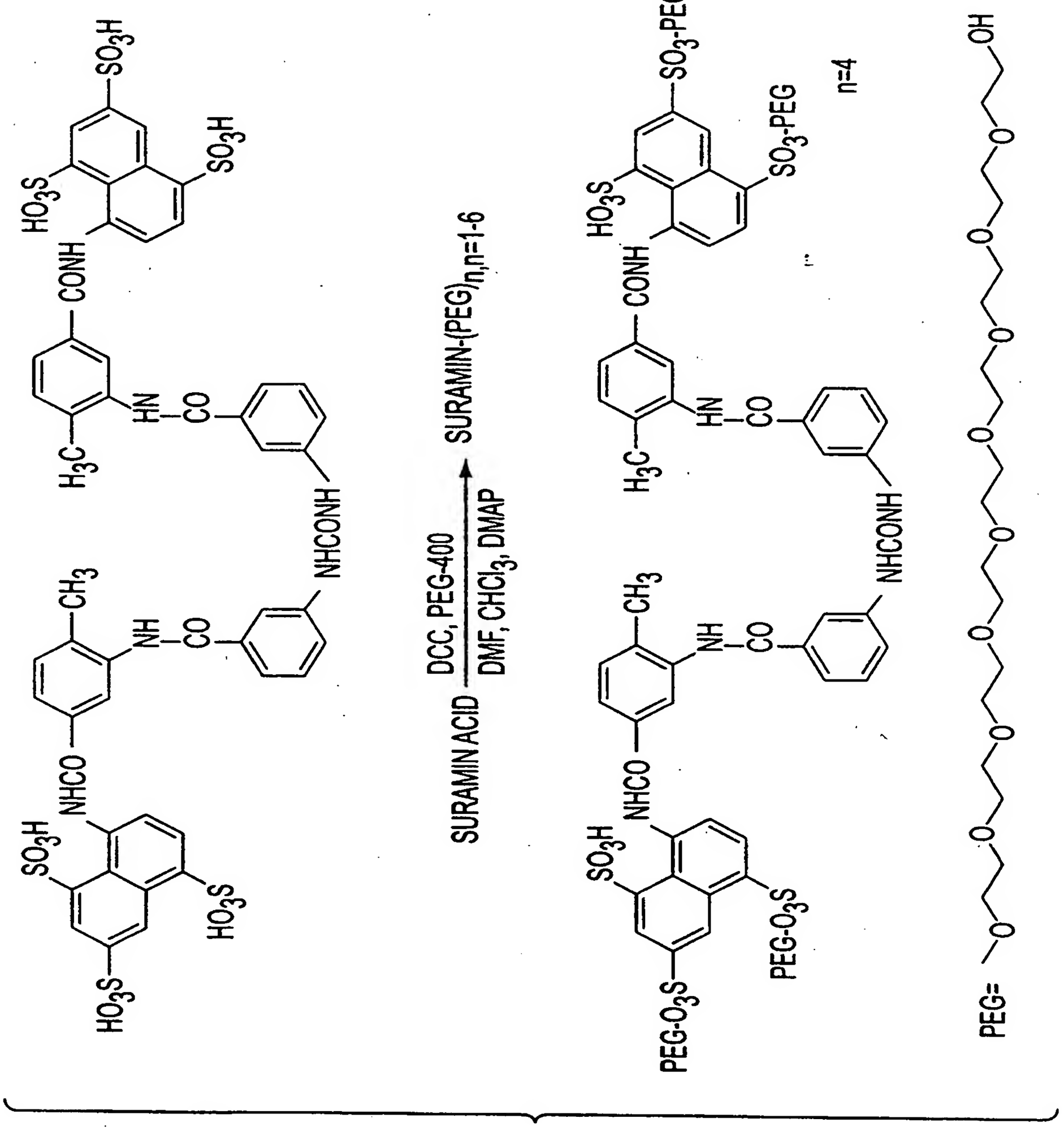

SURAMIN ACID
DCC, PEG-400
DMF, CHCl3, DMAP
SURAMIN-(PEG)n,n=1-6
n=4
PEG=

FIG. 4

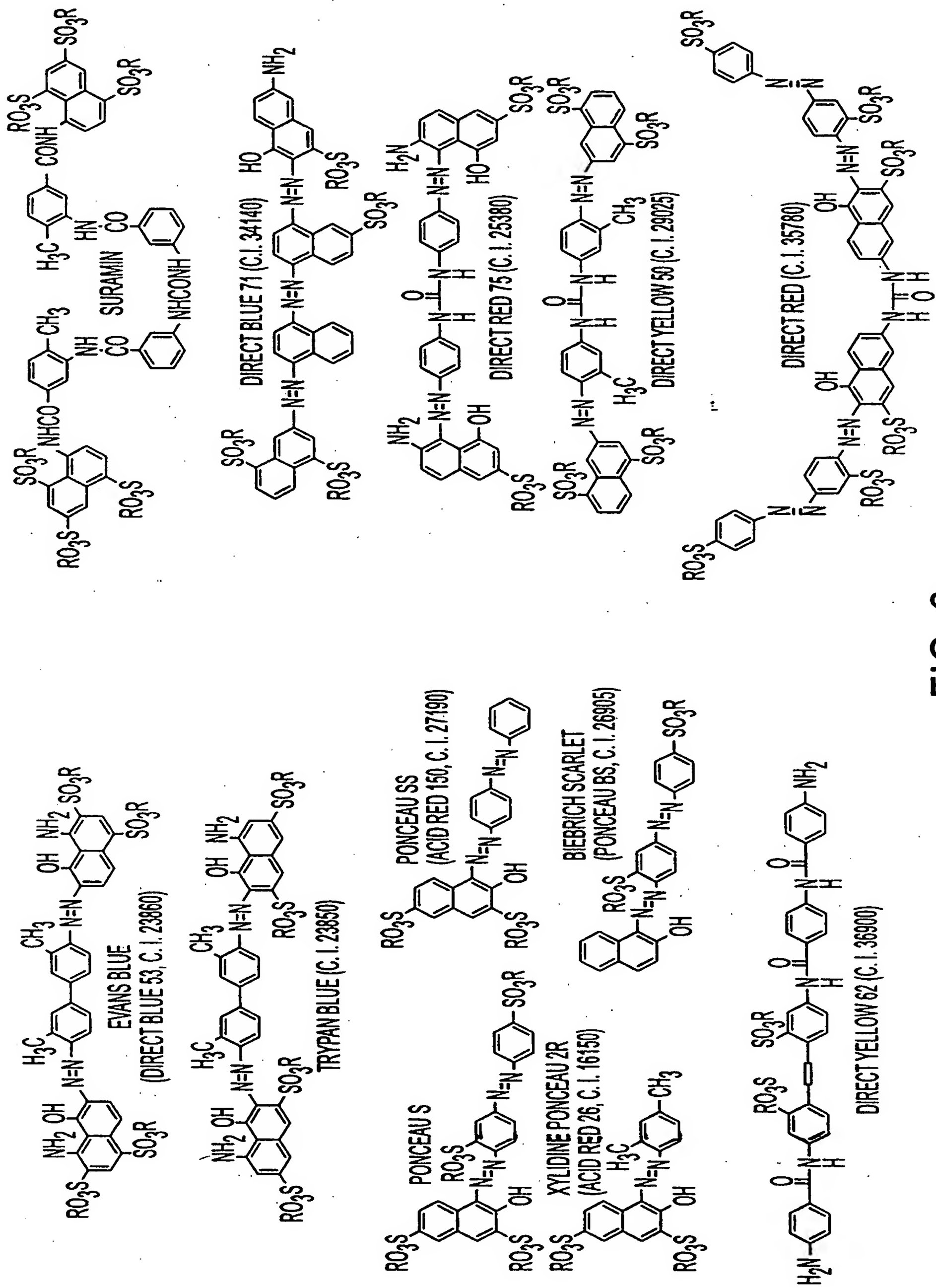

SURAMIN
DIRECT BLUE 71 (C.I. 34140)
DIRECT RED 75 (C.I. 25380)
DIRECT YELLOW 50 (C.I. 29025)
DIRECT RED (C.I. 35780)
EVANS BLUE
(DIRECT BLUE 53, C.I. 23860)
TRYPAN BLUE (C.I. 23850)
PONCEAU SS
(ACID RED 150, C.I. 27190)
BIEBRICH SCARLET
(PONCEAU BS, C.I. 26905)
PONCEAU S
XYLIDINE PONCEAU 2R
(ACID RED 26, C.I. 16150)
DIRECT YELLOW 62 (C.I. 36900)

FIG. 6

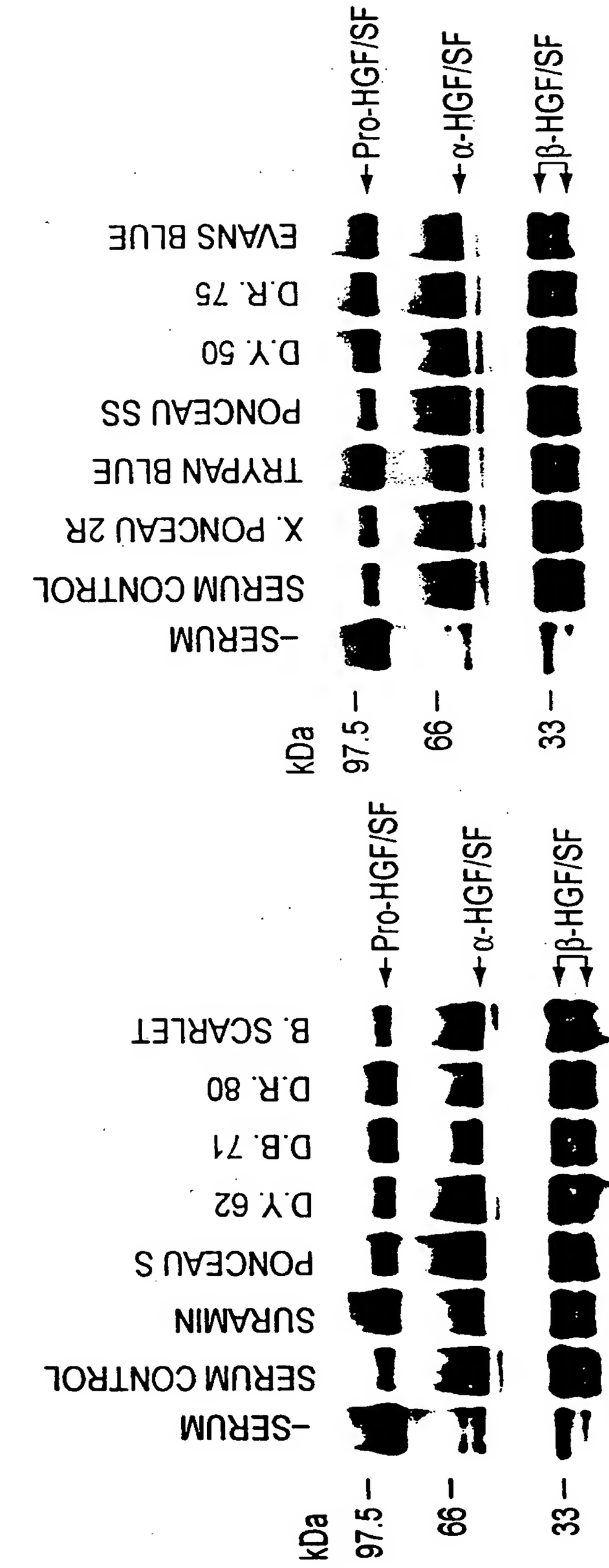


FIG. 7A

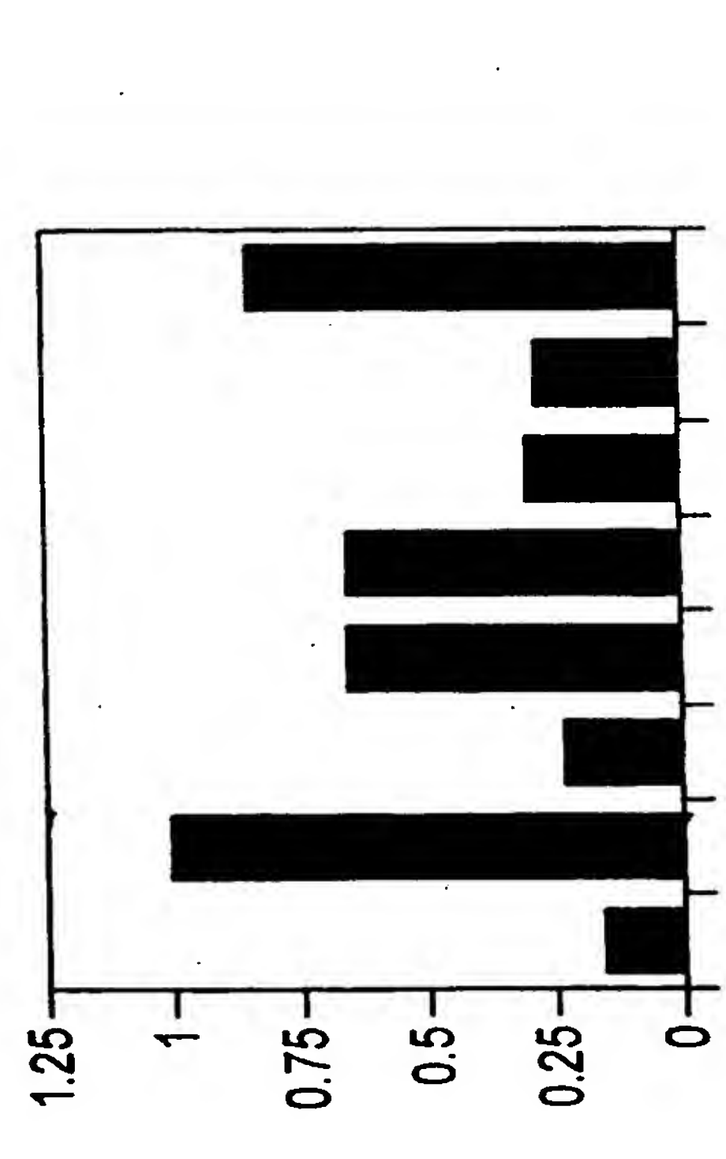


FIG. 7C

FIG. 7B

FIG. 7D

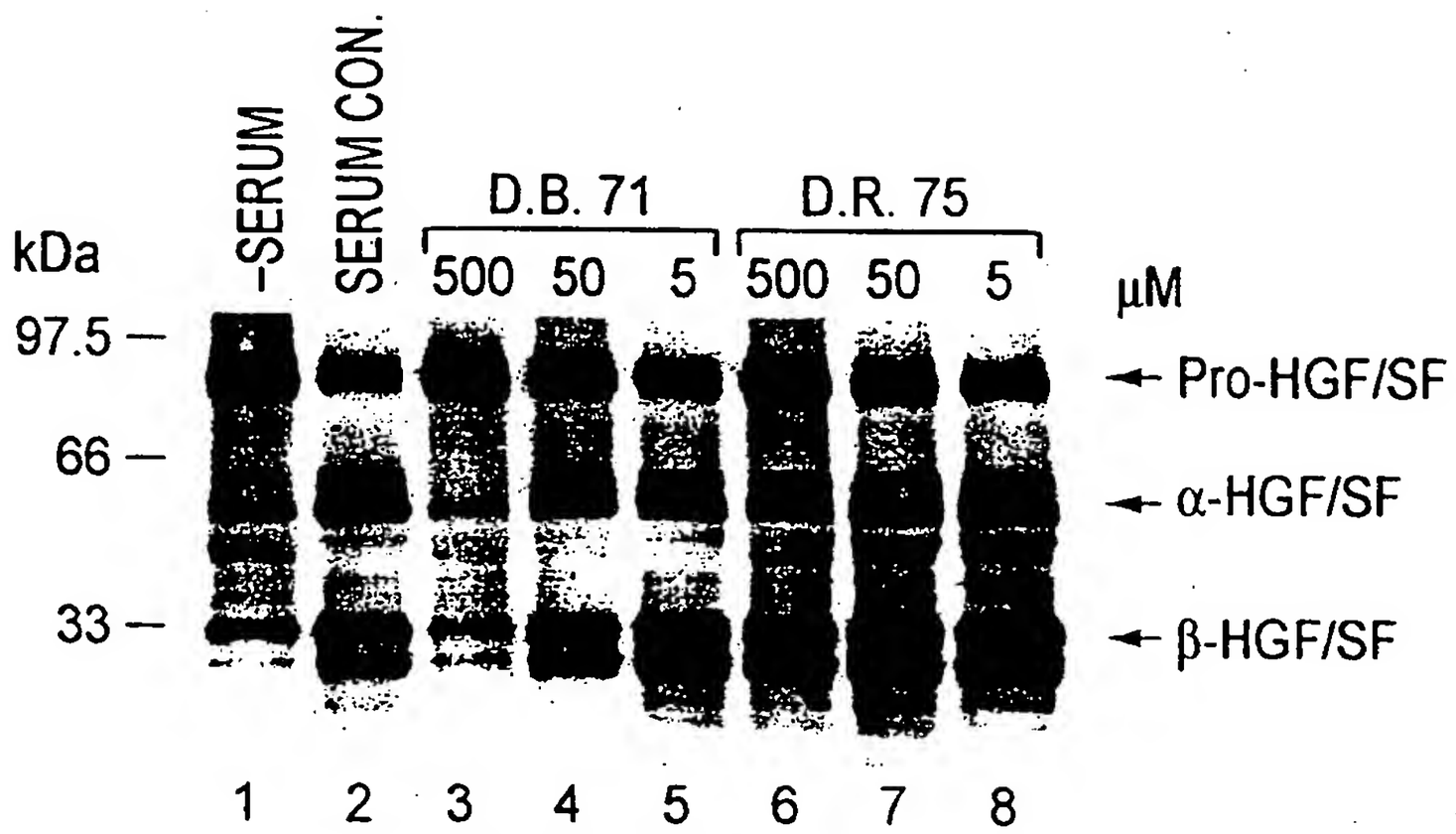


FIG. 8A

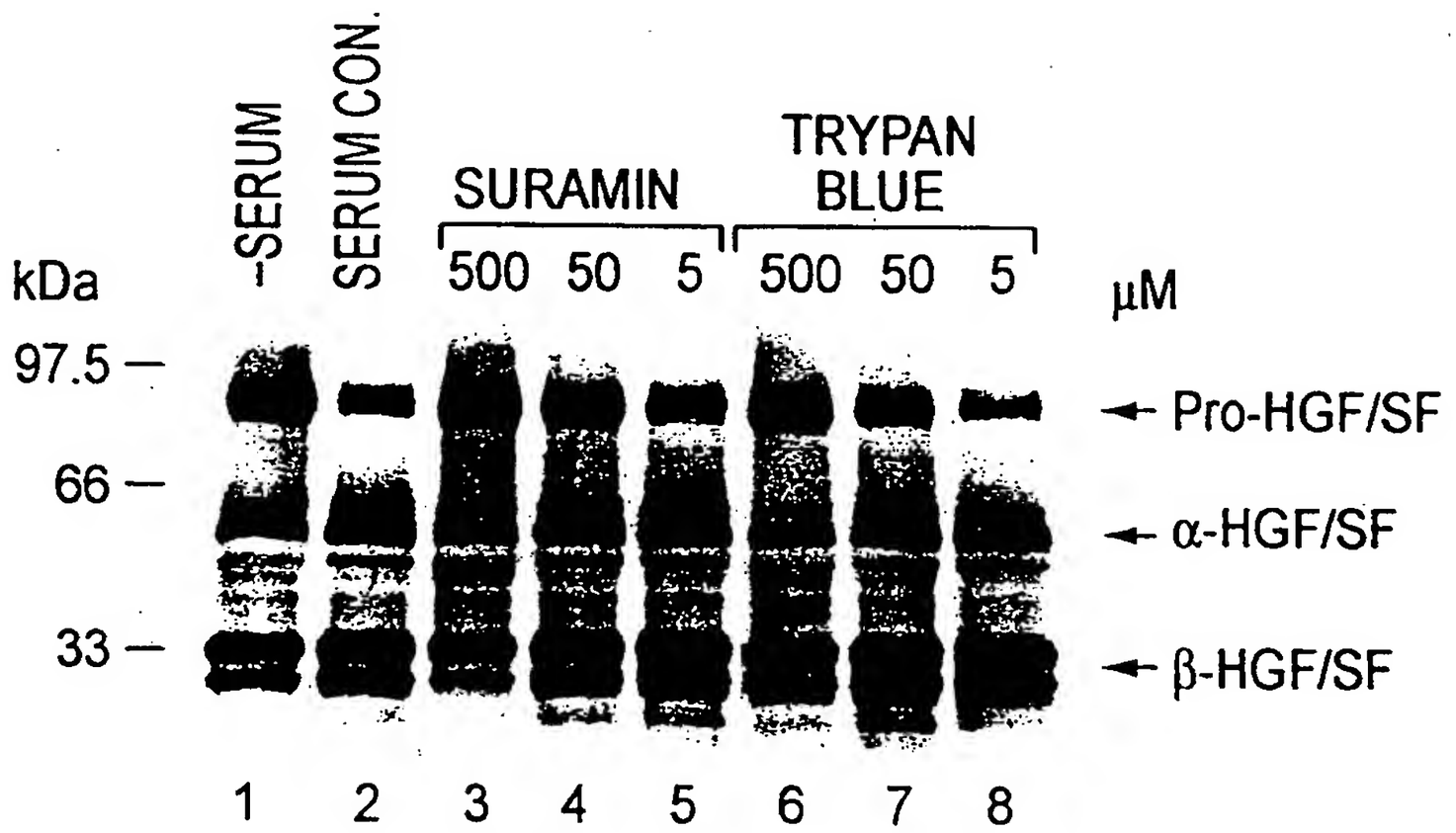


FIG. 8B

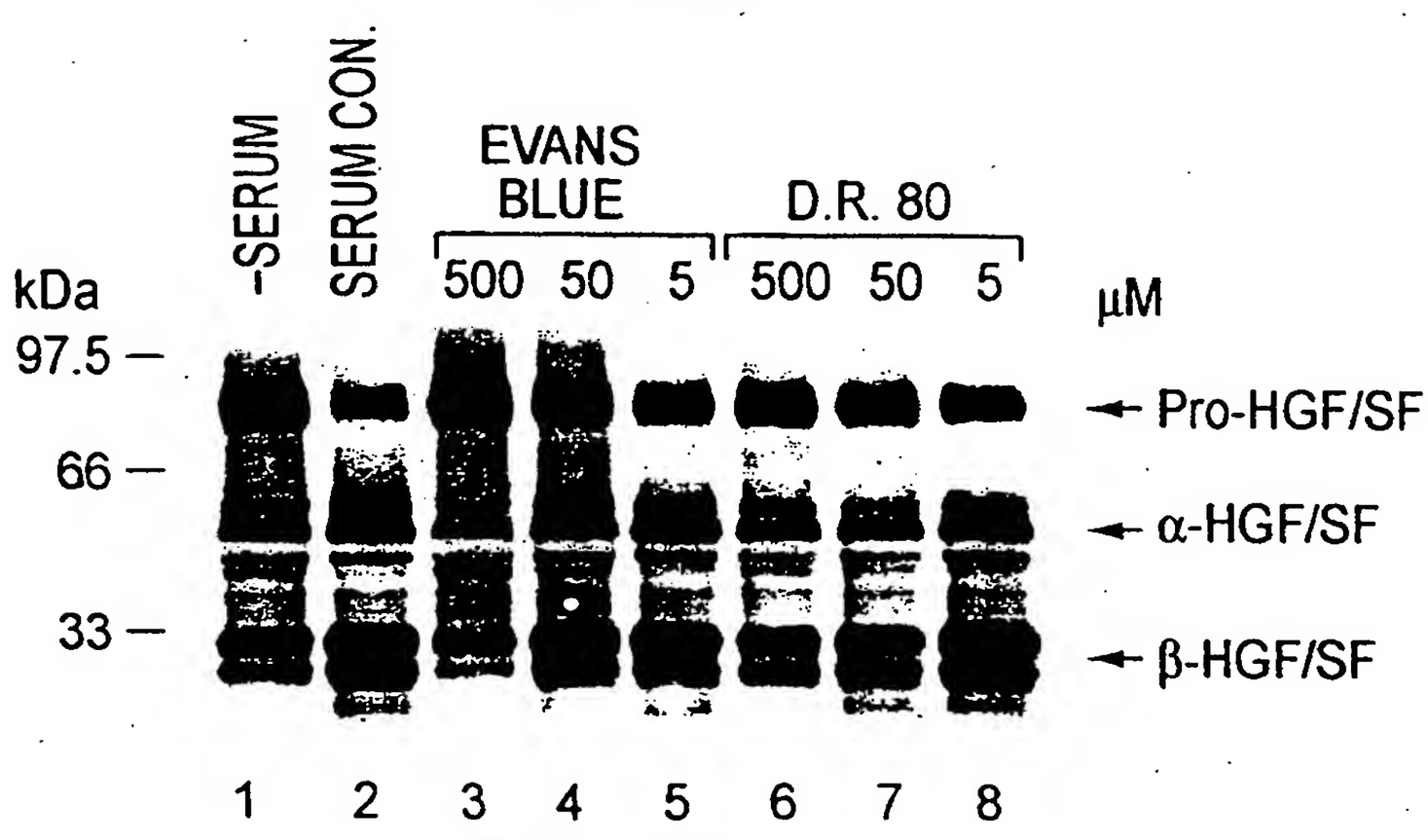


FIG. 8C

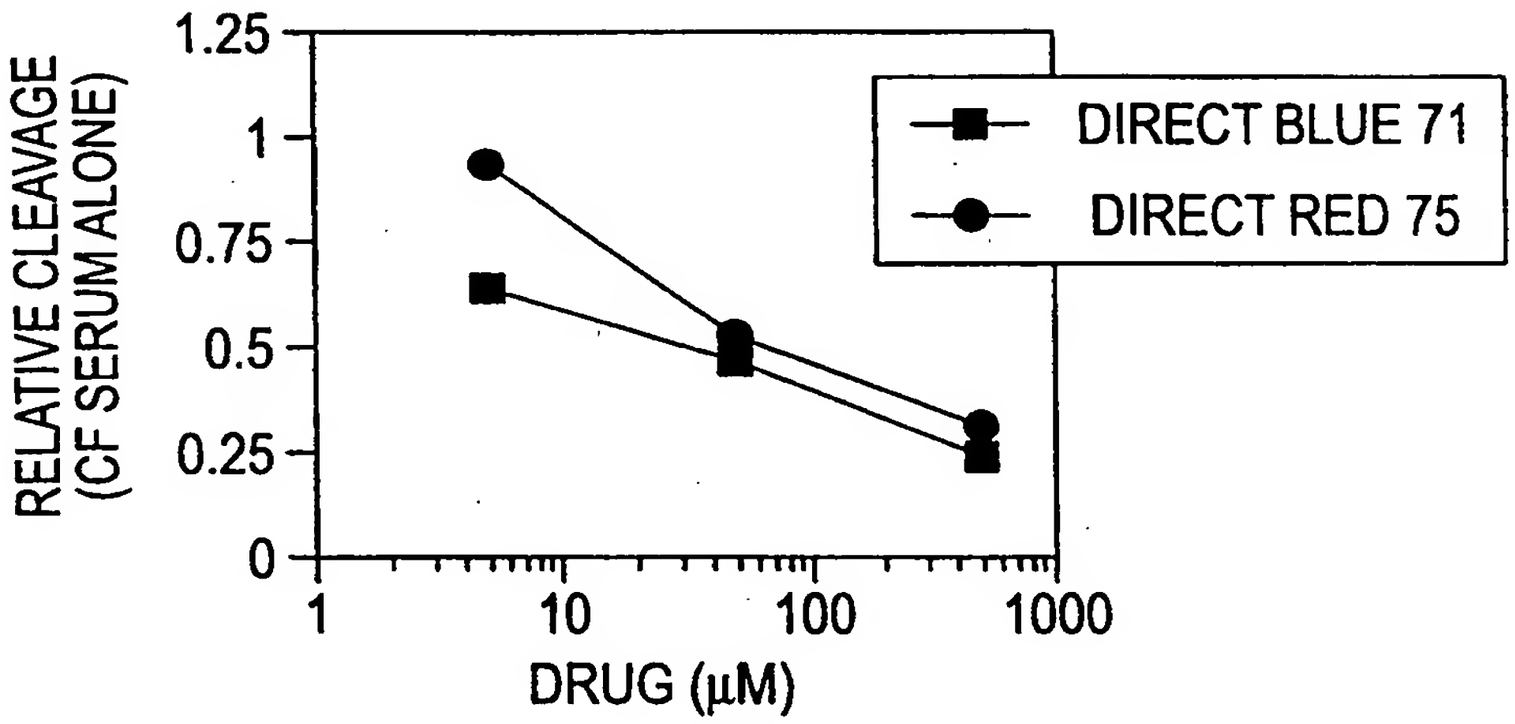


FIG. 8D

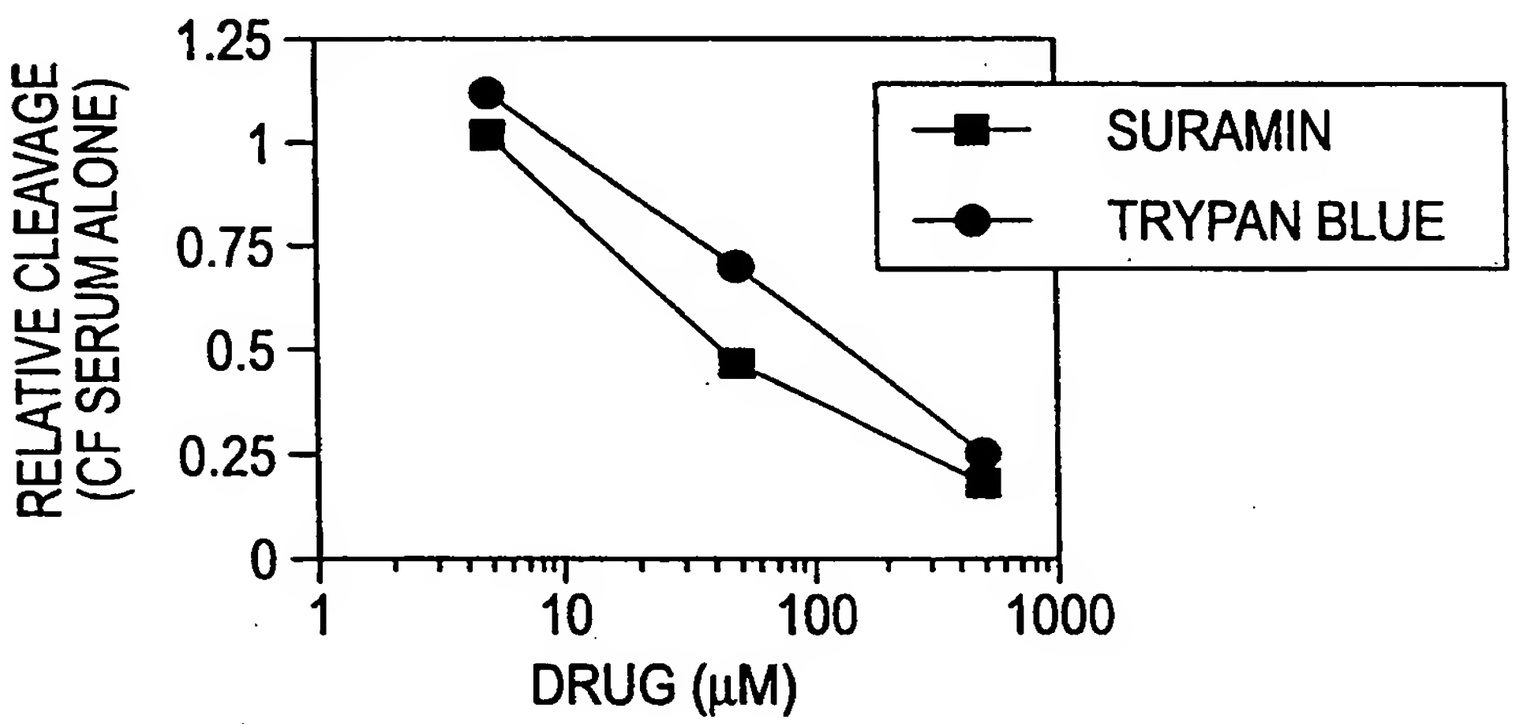


FIG. 8E

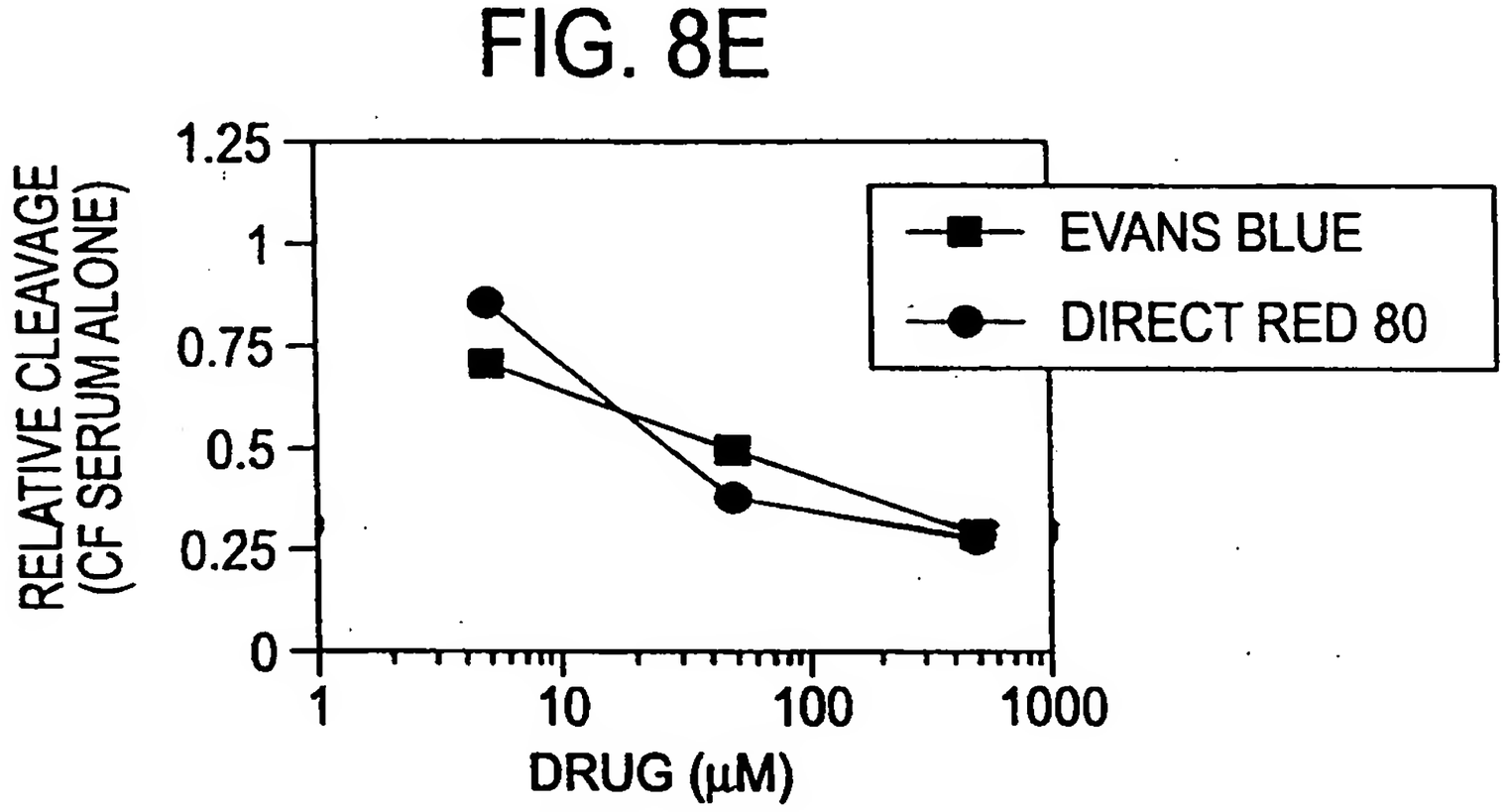


FIG. 8F

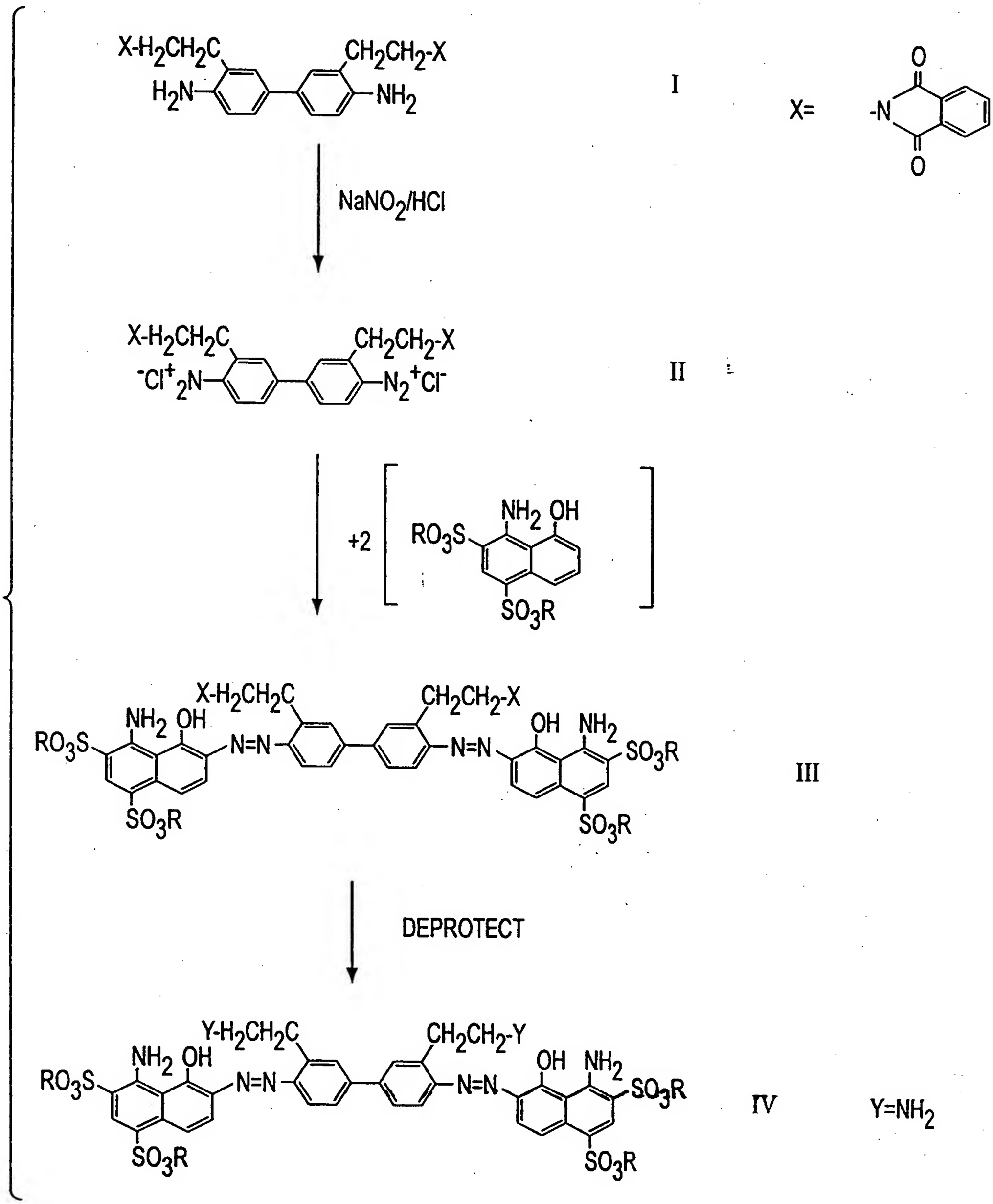


FIG. 9